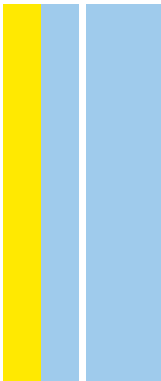


DISSERTAÇÃO DE MESTRADO
TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Cytotoxicity induced by extracts/
compounds of *Pisolithus tinctorius*
spores on human cancer and normal
cell lines - evaluation of anticancer
potential

Ricardo Manuel Gonçalves Alves

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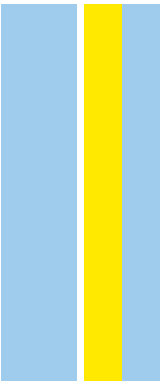
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**CYTOTOXICITY INDUCED BY EXTRACTS/COMPOUNDS OF
PISOLITHUS TINCTORIUS SPORES ON HUMAN CANCER AND
NORMAL CELL LINES - EVALUATION OF ANTICANCER
POTENTIAL**

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Mestre em Toxicologia e Contaminação
Ambientais submetida ao Instituto de
Ciências Biomédicas de Abel Salazar
da Universidade do Porto.

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Abstract

Cancer is classified as the greatest threat to human health in modern times. Currently conventional medicine uses chemotherapy as preferential treatment. However, this therapy entails acute side effects in patients, largely due to their lack of selectivity. Basidiomycetes are well known in the literature for the ability to produce secondary metabolites capable of being useful in different biomedical fields, including anticancer compounds. In a previous work, *Pisolithus tinctorius*, a globally distributed fungus capable of forming ectomycorrhizas in association with several trees, has already demonstrated a potential bioactivity with spores extracts and cancer cell lines. Following this work, the main objective of this dissertation was to optimize the extraction procedure of *P. tinctorius* spores, and following a bioassay guided fractionation to isolate compounds involved in the cytotoxicity. Initially two extractions were carried out only with solvent and sonication, resulting in a low extraction efficiency. With this, four different experimental methodologies were carried out to find the best extraction technique: extraction using only solvent (1), solvent and glass beads (2), solvent and sonication (3) and solvent, glass beads and sonication (4). Considering the results obtained, method 2 was selected for use in large-scale extraction. By vacuum liquid chromatography, using an increasing polarity gradient, it was possible to obtain 9 different fractions per extract. These fractions were tested for cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and using the cancer cell lines T47D, RKO, HEPG2 and the normal cell line HCMEC/D3. Fractions were tested at concentrations of 100, 10 and 1 $\mu\text{g}/\text{ml}$ over the 24 h, 48 h and 72 h. Due to the low mass recovered, the fractions that caused acute cytotoxic were grouped into a single pool, and then subjected to a gravity chromatography process (performed in parallel with thin layer chromatography), and 22 fractions were collected. Nuclear Magnetic Resonance analysis and cell viability assay of these fractions was performed to pooling the fractions that displayed the highest cytotoxic activity. This pool was subjected to high pressure liquid chromatography (HPLC), revealing a complexity, requiring further fractionation to identify and isolate the compound(s) responsible for this bioactivity. This study confirms the existence of

bioactive compounds with anticancer potential. The optimisation of the extraction process using glass beads is a first step in establishing new extraction techniques. However, further refinements are necessary to obtain sufficient mass, to be able to isolate and identify the compounds responsible for the bioactivity.

Resumo

O cancro é considerado como a maior ameaça para saúde humana nos tempos atuais. Atualmente a medicina convencional utiliza a quimioterapia como tratamento preferencial. Contudo esta terapia acarreta efeitos secundários nos pacientes, derivado em muito a sua falta de seletividade. Os basidiomicetes, são conhecidos na literatura pela sua capacidade de produzirem metabolitos secundários com potencial farmacológico nomeadamente potencial anticancerígeno. Num trabalho anterior, extratos de esporos de *Pisolithus tinctorius*, um fungo distribuído mundialmente e com capacidade de formar ectomicorizas em associações com diversas árvores, revelou potencial bioativo em linhagens celulares cancerígenas. Tendo em conta este trabalho o principal objetivo desta dissertação de mestrado foi otimizar o processo de extração com esporos de *P. tinctorius* e seguindo um fracionamento guiado por bioensaio isolar os compostos envolvidos na citotoxicidade. Inicialmente duas extrações foram efetuadas apenas com solvente e com a ação de um sonificador, resultando numa baixa eficiência de extração. Com isto quatro diferentes metodologias experimentais foram efetuadas: Extração com recurso apenas a solvente (1), uso de esferas de vidro com 3mm de diâmetro (2), solvente com auxílio de sonicação (3) e finalmente a extração com o recurso a esferas de vidro com diâmetro de 3mm, juntamente com sonicação (4). Tendo em conta os resultados obtidos, o método 2 foi selecionado para ser usado na extração em larga escala. Através de uma cromatografia líquida com vácuo, usando um gradiente crescente de polaridade, foi possível obter 9 frações distintas por extrato. Estas frações foram sujeitadas a um teste de citotoxicidade através de um ensaio com 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromado (MTT), com as linhas celulares cancerígenas T47D, RKO, HEPG2 e uma linhagem celular normal HCMEC/D3, em concentrações de 100, 10 e 1µg/ml durante o período de 24h, 48h e 72h. Devida a baixa massa recuperada, as frações que despertavam resultados citotoxicológicos agudos, foram agrupadas numa única *pool*, sendo posteriormente sujeitas a um processo de cromatografia por gravidade, (realizado paralelamente uma cromatografia de camada fina, sendo recolhidos 22 frações e observando o ensaio de viabilidade celular estas frações, agrupou-se as que apresentavam maior atividade citotóxica. Esta *pool* foi submetida a uma cromatografia líquida de alta pressão (HPLC), revelando uma complexidade, sendo necessário mais um fracionamento de maneira a poder identificar e isolar o

composto(s) responsáveis por esta bioactividade. Este estudo vem confirmar a existência de compostos bioativos com potencial anticancerígeno. A otimização do processo de extração com o recurso a esferas de vidro, representa um primeiro passo em estabelecer novas técnicas de extração. No entanto, novos aperfeiçoamentos são necessários, de maneira a obter massa suficiente, para assim ser possível identificar e isolar os compostos responsáveis por esta bioatividade anticancerígena.

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List of abbreviations

ATCC- American Type Culture Collection-

CDCl_3 – Deuteriochloroform

DCM- Dichloromethane

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

ECM- Ectomycorrhizal

hCMEC/D3- Human brain capillary endothelial cells

HIV/AIDS- Human immunodeficiency virus infection and acquired immune deficiency syndrome

HL-60- Leukemia cells

HPLC- High Pressure Liquid Chromatography

LDH- Lactate dehydrogenase

MeCN- Acetonitrile

MeOH- Methanol

Methicillin-Resistant *Staphylococcus aureus*

MTT- Dimethyl thiazolyl diphenyl tetrazolium

N- Nitrogen

NF- κ B- Nuclear factor kappa-light-chain-enhancer of activated B cells

NMR- Nuclear magnetic resonance spectroscopy

PBMC- Peripheral blood mononuclear cells

PSK- Polysaccharide-K

RKO- Human colon adenocarcinoma cell

ROS- Reactive oxygen species

SD- Standard Deviation

T47D- Human breast cancer cell line

TLC- Thin Layer Chromatography

USA-United States of America

VLC- Vacuum liquid chromatography

WHO- World Health Organization

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1. Introduction

1.1. Basidiomycota and chemical properties;

Basidiomycota are one of the big divisions of the higher kingdom of Fungi (Moore, 1998). In this group, we can include a vast number of types of Basidiomycota like mushrooms, puffballs, earthstars and other diverse organisms. Their ability to produce countless number of secondary metabolites aroused attention of the scientific community to discover the of these new compounds properties. The rise of synthetic substances and chemical syntheses did not stop the exploration of natural compounds. Furthermore, today, more than ever, the necessity to give response a wide range of problems, the search for secondary metabolites with biological activity happens at an explosive rate. Therefore, most of the new drugs created by pharmaceutical industries are from natural products, with 80% of this medicine having this origin (Wasser, 2014) (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969). To reinforce the importance of these compounds, drugs like antibiotics (penicillin and erythromycin), anti-obesity pharmaceuticals (lovastatin) and immunosuppressants (cyclosporin) have their origin in secondary metabolites of Basidiomycota

Distinctive societies use these organisms in their diet, given their marked taste and the ease of growth in very extreme environments. Furthermore, they can be composed up to 40% of protein of its dry weight, being an excellent source of amino acids, possessing a good nutritional value (Money, 2016). Due to these facts, the ancient civilizations use the basidiomycotas to treat health problems. Oriental societies were pioneers in use of the Shiitake (*Lentinus edodes*) and Reishi (*Ganoderma lucidum*) in the treatment of different cancers and immunodeficiency disorders. There are other reports in the use of the psychedelic mushroom *Amanita muscaria* in the Mesoamerican, Celtic and Russian cultures with purpose of spiritualism and to reach a higher level of conscience (Chang & Miles, 1989). However, only with the advent of the chemical syntheses and the development of the biochemistry, the researchers started to get insights of the mechanisms of actions of these compounds. In 1969, it was isolated a polysaccharide, named Lentinan from *Lentinus edodes*, showing a strong anticancer activity in mice sarcoma 180. The isolation of this compound led researchers to look to the basidiomycota as new potential source of anticancer drugs.

As one of the deadliest disease, cancer is still draw the scientific community to find new approaches to fight this illness. In fact, the World Health Organization (WHO) reports that cancer is the main cause of mortality in 1 in 6 dead's and is expected the number of oncological patients rise by 70% until 2030.(McGuire, 2016)

Traditionally, the western medicine relies on chemotherapy as a way to treat cancer. However, most of the drugs used in this technic are cytotoxic substances, with a lack of selectiveness, targeting the normal and the oncological cells to induce the apoptosis. As consequence of this aggressive therapy, most patients suffer from side effects with immunosuppression, opening a door to opportunistic infections(Patel & Goyal, 2012). Nausea and acute pains are usually common, along with an impact in the patient self-esteem. Most of the drugs with origin in mushrooms are use as complementary of chemotherapy and radiotherapy, mostly to stimulate immune system to attack the cancer cells and preventing the infections from other diseases. Following this problematic, the modern pharmaceutical industry is trying to discover new compounds with different mechanisms of actions of the *mainstream* drugs. The lucrative market of the cancer drugs has reallocated most of the funds to the research of the innovative therapy's with experience of the oriental cultures of medical mushrooms (Wasser, 2014). To reveal the importance, several drugs with origin in mushrooms are covered by the Japanese Health System. In 1993 25 % of anti-cancer drugs in the Nippon market had origin in mushrooms (Mizuno, 1999). Other study reports that workers from edible mushroom farm in Nagamo District (Japan), and consequently their diet were based in this Fungi, had a much lower rate of cancer incidence than the general population. (Ikekawa, 2001).

The most studied are the β -Glucans, a type of β -D-glucose polysaccharides, present in the cell wall in different organisms, including fungi. Most of this β -Glucans are chains glucose polysaccharides connected by β -type glycosidic bonds (Lemieszek & Rzeski, 2012; Ren, Perera, & Hemar, 2012). Proteins can be connected in the side chains creating molecules like polysaccharide-K (PSK). This polysaccharide is extracted from a basidiomycota called *Trametes versicolor* and some studies show the potential of PSK to be used as adjuvant in the treatment of several cancers like leukaemia, breast and liver cancer. This compound is able to stimulate the instigation of natural killer cells, T-cells, dendritic cells and induce the production of cytokines. (Fritz et al., 2015).

Although β -Glucans are well known in literature, secondary metabolites produce by the fungi can have a beneficial effect rising the interest from the therapeutic view. Compounds like terpenoids, triterpenoids, lactones and alkaloids have been identified

has potential anti-cancer drugs. (Petronelli, Pannitteri, & Testa, 2009). Some of these compounds are capable to interfere with intracellular pathways, like panepoxydone, Extracted from *Lentinus crinitus*, panepoxydone can inhibit the DNA binding of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Arora et al., 2014; Erkel, Anke, & Sterner, 1996). The deregulation of this molecular mechanism is a characteristic of many cancers and compounds inhibitors of NF-κB can be used to mediate inflammatory process and hold the cancer cells proliferation. (Sethi, Sung, & Aggarwal, 2008). Moreover, enzymes with antioxidant potential has been documented, such as superoxide dismutase, glucose oxidase and peroxidase. The presence of these enzymes can help the cells to deal with reactive oxygen species (ROS's), highlighting the importance of the fungi to prevent damages from oxidative stress. (Wei, Helsper, & Van Griensven, 2008; Wei & Van Griensven, 2008).

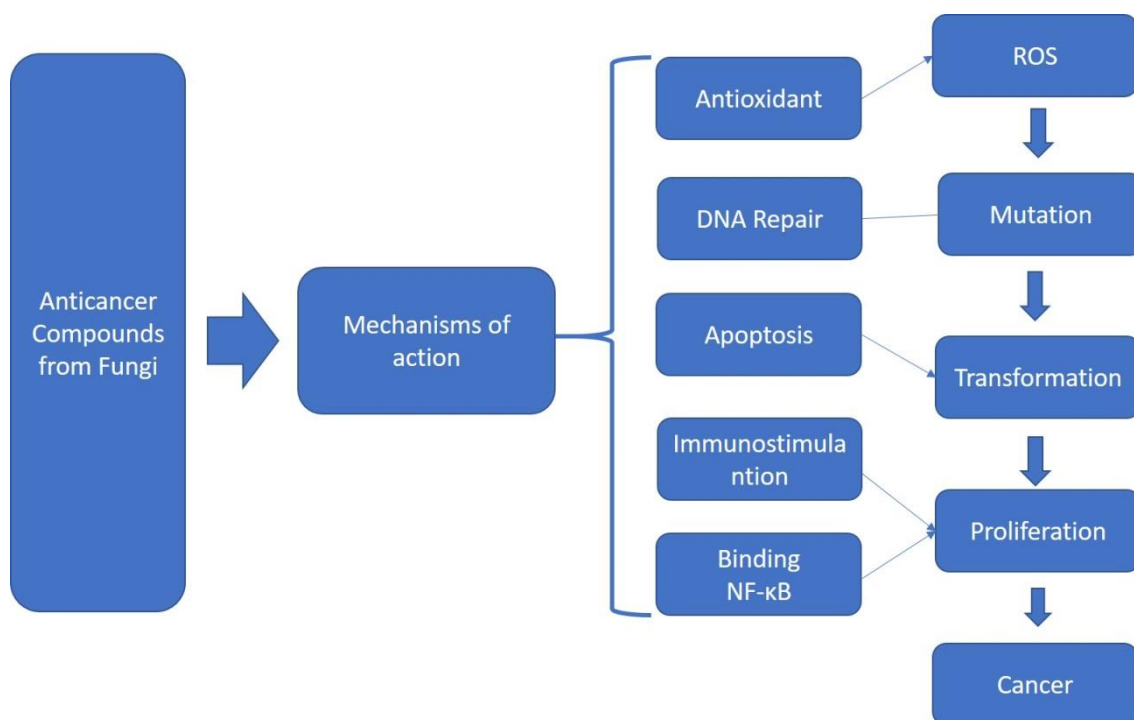


Figure 1-Mechanisms of action from the anticancer compounds from fungi (Wasser, 2014)

These compounds can be obtained from different parts from the fungi with diverse extraction processes, being available to the pharmaceutical market: (Wasser, 2014).

1. Extracts from freeze dried fruit body powders.
2. Extracts from freeze dried mycelium;

3. Biomass or extracts from mycelium from liquid culture grown in a bioreactor;
4. Spores and their extracts;

The constant demanding for new compounds with bioactive response put some pressure on pharmaceutical industry. In this perspective, Wasser, 2014 established a protocol called Drug Discovery Procedure, to put a standard process to discover new therapeutic drugs.

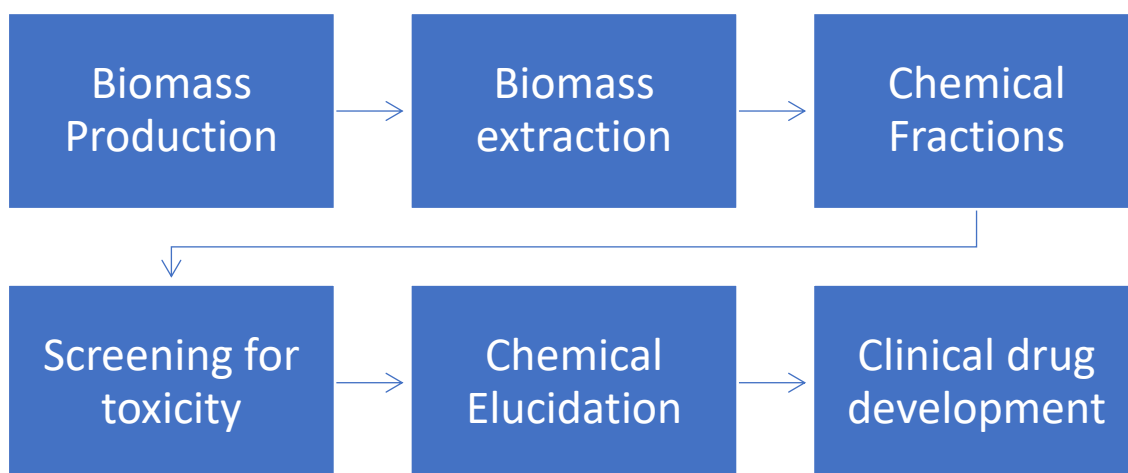


Figure 2. The bioassay-guided fractionation process (Wasser, 2010; Weller, 2012)

Although these results are accepted in Eastern world, the scepticism in the Western Medicine can be explain by the rigid regulation enforced in these countries (Wasser, 2014) The lack of standardization and safety protocols can jeopardize the safe use of these compounds. The absence of information, leads Wasser, 2014 to also question if the bioactive effects are caused by an isolated compound or a group of them with a synergistic action. Other highlighted problem is the possibility of the compounds extracted from fungi interact with common used drugs, leading to unknown side effects.

1.2. *Pisolithus tinctorius*;

Pisolithus tinctorius (Pers.) Coker & Couch 1928 belong to basidiomycota phylum and to the Sclerodermataceae family. They have the ability to establish a symbiotic relationship with host trees, boosting their grow and development (Marx, 1977). The structure of *P. tinctorius* can be divided in two main regions: sporocarp, the zone connected to the soil, with a hard and rigid texture; and an upper zone with soft tissue called pseudoperidioles, containing a brownish pigment and the spores of this fungus. This organism can reach 20 cm of diameter,(Coker & Couch, 1928) weight up to 1 kg, with the spores size range between 7 to 11 μm (De Zubiria, Horner, & Lehrer, 1990). Due to large spectrum of heterogeneity in the morphology and with the help of DNA sequence data, some authors question the actual classification (Cairney, 2002). Furthermore, the results from (Martin, Díez, Dell, & Delaruelle, 2002), state that *P. tinctorius* is not a so called “*pan-global super fungus*”, but a complex of multiple species with more restrict geographical presence and limited host.

The ectomycorrhizal relationship is also essential to invasive tree species to colonise new habitats, with the prominent cases being the introduction of eucalypts in the Iberian Peninsula (Díez, 2005) or the invasion of *Pinus* species in Australia.(Richardson, 2000). In both cases, *Pisolithus* played a big role in contributing to the success of the invasion.

Highly used in reforestation, ectomycorrhizal fungi, such as *P. tinctorius* are key to the success of silviculture programs. The formation of fungal hyphae permits a deeper penetration in the soil allowing more efficient uptakes of N and transport to the host (Oliveira, Franco, & Castro, 2012). (Peay, Bruns, Kennedy, Bergemann, & Garbelotto, 2007) stated that the fungi can provide 86% of the N host need's and receive 15% of carbohydrates. Nutrients are not the only benefit the host can receive. Since the ectomycorrhizas have a tolerance to high concentration of heavy metals, *P. tinctorius* showed a potential to be use for bioremediation of contaminated soils (Tam, 1995). The formation of the hyphal net around the host roots can serve of physical barrier preventing predators and pathogens to attack the tree. *P. tinctorius* have an important role in producing compounds with anti-fungal and anti-bacterial activity providing an extra protection. (Tsantrizos, Kope, Fortin, & Ogilvie, 1991).

This result, highlighted the possibility of use the secondary metabolites in diverse fields of the biomedical sciences, boosting the research on understanding all bioactive potential of *P. tinctorius*.

1.3. Bioactive potential of *Pisolithus*

As was stated before, nowadays the main potential of *P. tinctorius* is centrally in the economic value due to the ability to boost reforestation programs. This interaction host-fungi increases the concentration of certain compounds, as terpenes, in the host whose function is to protect it against fungal infections (Abreu, 1987). For this motive, studies were done to verify the presence of allelochemicals, in other words, secondary metabolites that can influence the growth of other organisms. The presence and the understanding of these compounds in *P. tinctorius* can open doors to new approaches in biomedical fields as the constant resistance to antibiotics and the search for new compounds, to be used as fungicides, explore the possible potential as antioxidant or even inhibit the grow of cancer cells as was demonstrated by Montenegro et al., 2004

1.3.1. Anti-cancer potential

As was describe before, cancer is considered of the most problematic disease that we face off. The actual therapies had demonstrated low efficiency with high collateral effects for the patients. Consequently, there has been a constant focus and growing interest from researchers to find new useful compounds to fight this infirmity. To verify the anticancer potential of *P. tinctorius*, some studies were made, especially with pisosterol, a compound primarily isolated by Gill, Kiefel, Skelton, & White, 1989, which is a triterpene present in the sporecarp of the fungus.

Montenegro et al. in 2004 conducted the first study concerning the anticarcinogenic action of pisosterol. This triterpene was tested *in vitro*, in erythrocytes of rats, sea urchin's eggs and in the human cancer cell lines, such SF-268, B16, PC-3, MCF-7, HCT-8, HL-60 and CEM. By recurring to MTT assay, the authors were able to verify the mitochondrial activity of the cancer cell lines. Data revealed, membrane disruption in mouse erythrocytes was not observed and pisosterol has not interrupted the development of the sea urchin eggs. However, there was a strong growth inhibition in

all cancer cell lines, more notably in leukemia and melanoma cells. The same authors suggests that the selectivity have a different mechanism of action than the common cytotoxic drugs.

With this data, Montenegro and his team in 2007, conducted a study to determine if pisosterol was able to induce differentiation using HL-60 cell lines (leukemia cells) and normal cells peripheral blood mononuclear cells (PBMC). The data showed that HL-60 cells treated with pisosterol tended to differentiate into monocytic cells. Apoptosis was detected in HL-60 cells exposed to pisosterol, despite no cytotoxicity was observed in PBMC cells, even in the highest concentration (5 µg/ml), suggesting that pisosterol can be selective to cancer cells lines and revealing the potential of this compound to be used in the cancer treatment. This study was determinant since it is essential to evaluate the damages in the dividing proliferating lymphocytes, to prevent collateral damages. (Anazetti, Melo, Durán, & Haun, 2003)

The cancer cell HL-60 genome has a proto-oncogene called *C-MYC*, located in the homogeneously staining region (HSR) 8q24 aberration and has an important function on regulating the cell life cycle. It is known that, cancer cells with low degree of expression of *C-MYC* are less aggressive and so, more susceptible to chemotherapy treatment. (Eckhardt et al., 1994; Fegan, White, & Sweeney, 1995). It was observed that 90% of the analysed cells lacked this HSR region when threatened with pisosterol. Consequently, these results opened the possibility of the use of pisosterol combined with chemotherapy, since this compound has the ability to block aggressive mechanisms of certain cancers.

Pisosterol was also tested in human glioblastoma cell lines. (Pereira et al., 2011) These type of cancer cells are known for their acute aggressiveness with an unfortunately, average rate survival of one year after being detected (Pinto et al., 2009). The study used two different cell lines, U343 and AHOL1, and before the treatment, 72% of U343 and 65% of AHOL1 cells registered more than two alleles of *C-MYC*. By being exposed to 1.8 µl/ml of pisosterol, only 33% of U343 cells and 15% of AHOL1 cells showed signs of containing *C-MYC*, signifying that pisosterol also blocks the expression of this proto-oncogene.

Since there was no data regarding the anticancer activity of pisosterol in *in vivo* assays, Montenegro et al., 2008 conducted a study where Sarcoma 180 tumours were transplanted to Swiss female mice and treated with 50 mg/m² and 100 mg/m² of pisosterol. The results showed that this compound was able to inhibit tumour growth by 43%. Morphological exams were made to evaluate the influence of pisosterol on the

mice organs. The treatment had an impact in the liver, showing kupffer cells hyperplasia, focal infiltrate of inflammatory cells, centrilobular venous congestion, demonstrating that the liver is a target organ of pisosterol. Although, the authors concluded that the damages can be reversible since no stromal fibrosis was detected and conjunctive tissue was preserved.

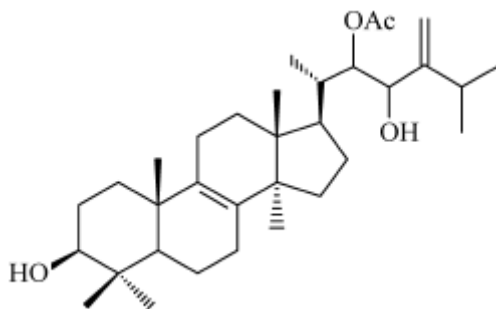


Figure 3. Chemical structure of Pisosterol

Most of the studies that were done, have use the mycelium or the sporecarp to perform extraction, ignoring other structures of *P. tinctorius* such as the spores. The inoculation of the spores, through an allergy skin test, had provoked an allergy symptoms in 9% of the subjects (De Zubiria et al., 1990). Workers from silviculture industry and college students were subjected to the skin prick test with spores extracts. The workers group was the most affected, with 28.2% showing positive reactions, and 82% of individuals from these positive tests developed symptoms of respiratory allergy. The authors stated that, since this basidiomycete is used to boost reforestation, the exposure to spores can be considered a possible occupational hazard by provoking respiratory allergic asthmas.

Considering the absence of data in the literature, regarding the bioactivity potential of the *P. tinctorius* spores, (Alves, Preto, Vasconcelos, Oliveira, & Martins, 2015) conducted a work to assess the presence of anticancer substances. Firstly, it was evaluated which solvent had the major yield extraction and less mitochondrial activity. The result showed that the mixture of DCM:MeoH (2:1) had the best yield considering the mitochondrial activity. Starting from the crude extract, through successive bioassay-guided fractionation, 11 fractions were screened for cytotoxicity in MG63 (bone osteosarcoma), T47D (human breast tumour), RKO (rectal carcinoma) cell lines and simultaneity with normal endothelial cells. The data revealed a decrease in cancer cell

viability with a low impact in the normal cells. On the other hand, considering the very low yield of extraction, it was not possible to purify, identify and isolate the compound(s), responsible for the anticancer response. So Alves et al., 2015 suggested in further studies, improving the extraction technique to help isolate the active compound.

1.3.3. Antimicrobial Potential

The discovery of penicillin has opened a new era in modern medicine, giving us the possibility to fight a large spectrum of diseases caused by bacteria (Golkar, Bagasra, & Pace, 2014). The massive use of antibiotics has led us to an increasing resistance of the bacteria to these compounds. Some authors state this is probably the biggest threat to the human public health in the XXI century (Ventola, 2015). The statistics shows that more people die in USA of diseases provoked by methicillin-resistant *Staphylococcus aureus* (MRSA) than HIV/AIDS, Parkinson's disease, emphysema, and homicide together. (Golkar et al., 2014; Rossolini, Arena, Pecile, & Pollini, 2014). Therefore, it has been an intense research to find new compounds that demonstrate efficiency to fight the so called *super bugs*, and fungi are a promising source for this type of drugs.

The first report of antimicrobial activity from *P.tinctorius* came from Kope and Fortin in 1989, when antifungal activity in cell free cultures was observed, provoking hyphal lysis to a different organisms. To understand the mechanism behind this activity, Tsantrizos et al., in 1991 isolated the pisolithin A and pisolithin B from *P.tinctorius* mycelium. . Although these metabolites were known in the literature, there was no data about their antifungal potential. These compounds showed a great capacity to reduce the mycelial growth of several phytopathogenic fungi such as *Rhizoctonia praticola*, *Truncatella hartigii*, *Sphaerospora brunnea*, *Fusarium solani*, *Brunchorstia pinea* and dermatopathogenic like *Microsporum gypseum* and *Trichophyton equinum*. The authors noticed also that the inhibition was similar, and in some cases higher than antifungal chemical agents such as nystatin and polyoxin D.

Tackling the problematic of the resistance of the bacteria's to the antibiotics, Ameri, Chanchala, Vaidya, & Deokule in 2011, conducted a study with crude extract and isolated fractions of *P. albus* against strains of MRSA. The collected fungi were associated with *Acacia auriculiformis* and *Eucalyptus globulus* trees. The crude was extracted with ethyl acetate, methanol and water and the fractions were from zones of

diterpenoids, triterpenoids, sesquiterpenoids, and polysaccharides I, II, IIIA and IIIB. The results showed that the crude extract with ethyl acetate and methanol had a strong antibacterial activity. Likewise, the fraction containing sesquiterpenoids displayed a robust action with the minimal inhibitory concentration determined to be in a range of 0.62 to 1.2 mg/ml. The author declared that with these results there are potential to the secondary metabolites of *P. albus* be use as antimicrobial drugs against certain strains of resistant bacteria. It also interesting to highlight the fact of *P. albus* collected from *Acacia auriculiformis*, did not show any pronounced activity against the majority of MRSA strains, proposing the possibility of that different hosts can induce the production of diverse compounds. More recently, an assay was conducted with extracts of eight species of ECM fungi (including *P. albus*) for tested anti-fungi potential against nine types of plant pathogenic fungi. It was observed that *Pisolithus* had an inhibitory effect in average 33.27% of those plant pathogenic fungi.(Mohan, Nivea, & Menon, 2015a)

1.3.5. Other bioactivity potentials:

Regarding the basis that supports the possibility of an immunosuppressive potential in fungal compounds, (Fujimoto, Nakayama, Nakayama, & Yamazaki, 1994) was responsible for isolating and characterising the components, using three different species of mushrooms, including *P. tinctorius*. With this fungus, he extracted compounds such as pisolactin and ergosterol peroxide, where the IC₅₀ was evaluated in relation to the proliferation of lymphocytes in rat spleens stimulated with concanavalin A and lipopolysaccharides.

Additionally, in 2011, the antioxidant potential was also unveiled. In aerobic organisms, free radicals are constantly being produced during natural cell metabolism, and there are compounds that can counteract undesirable forms of active oxygen in the body, detoxifying it. For that effect, (Reis, Ferreira, Barros, & Martins, 2011), did a comparative study between tocopherol composition and the antioxidant properties of the fungus in vivo and in vitro, using fruiting bodies and mycelia respectively, obtaining as a result a high antioxidant capacity resulting from *P. tinctorius*, mainly when using the corresponding structure of mycelia (Reis et al., 2011))

Extract/Compound	Fungal structure	Fungal species	Assay	Biological activity	Reference
Pisosterol	Mycelium; Sporecarp	<i>P. tinctorius</i>	MTT (Montenegro <i>et al.</i> 2004; Montenegro <i>et al.</i> 2007) NBT reducing activity (Montenegro <i>et al.</i> 2007) Naphthyl acetate esterase activity (Montenegro <i>et al.</i> 2007) Antiproliferative effect (inhibition of DNA synthesis) Montenegro <i>et al.</i> 2007) Mitotic index (Burbano <i>et al.</i> 2007;Pereira <i>et al.</i> 2011) In vivo assay (Montenegro <i>et al.</i> 2008)	Anticancer	(Montenegro et al., 2004) (Montenegro et al., 2007) (Burbano et al., 2009); (Carvalho Montenegro et al., 2008); (Pereira et al., 2011)

Pisolithin A & Pisolithin B	Mycelium	<i>P. tinctorius</i>	Spore Inhibition	Antifungi	(Tsantrizos et al., 1991)
Pisolactona	Sporecarp	<i>P. tinctorius</i>	Proliferation of mouse spleen lymphocytes stimulation with concanavalin A Lipopolysaccharide	Immunosuppressive	(Fujimoto et al., 1994)
Crude extracts	Sporocarp	<i>P. albus</i>	Well agar diffusion method	Antibacterial	(Ameri et al., 2011)
Crude extracts	Mycelium	<i>P.albus</i>	Well agar diffusion method Chitinase activity	Antibacterial	(Mohan, Nivea, & Menon, 2015)
Crude and Fraction extracts from spores	Spores	<i>P. tinctorius</i>	MTT Assay	Anticancer	(Alves et al., 2015)
Mycelium and Spores extracts	Mycelium; Spores	<i>P.tinctorius</i>	Prick-test	Allergenicity	(Chavasco et al., 1997)

Tocopherols	Mycelium; Sporocarp	<i>P. arhizus</i>	DPPH radical-scavenging Activity Reducing power and inhibition of b-carotene bleaching	(Reis et al., 2011)
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Table 1 *Pisolithus* compounds known for having bioactive properties

2. Aim and outline of this dissertation

All data collected until now supports the possibility of *P. tinctorius* to be used as a source of compounds with interesting bioactivity properties. Most of the studies were done with sporocarps as the source of fungal material, leaving the bioactive potential spores unexplored. The work performed by Alves et al. (2015) opened a door to explore this part of the fungus, more precisely its anticancer potential. Considering this, and the study executed by the main objective of this master thesis was to improve the extraction method from *P. tinctorius* spores, to obtain higher yield efficiency and to obtain more data about the compounds responsible for the cytotoxicity.

3. Materials and Methods

3.1. Collection of *Pisolithus tinctorius* spores

In order to obtain samples of *P. tinctorius*, the method used by Alves et al. (2015) was followed. Fungi were collected from a forest ecosystem located in the north of Portugal, well known for the presence of this species. The *P. tinctorius* specimens were cut into small pieces, and the pseudoperidiole was lyophilised for 48 h, to dry the samples. After lyophilisation the spores were isolated with a help of a sieve with a pore of 1 mm.

3.2. Crude extraction and fractionation

Two extractions were performed following the method used by Alves et al., 2015. The spores were extracted with Dichloromethane-Methanol (2:1), through a sonication process. They were given the code names of PT01 and E15094. The conditions of the crude and fractionation are described in the table 5.

3.3. Improving the extraction technique

The low extraction yield with *P.tinctorius* spores obtained by the procedure already described, led to the necessary improvement of the method. In this sense, a step that uses glass beads was included. The use of glass beads to lyse cell walls and membranes is extensively used, as in the DNA extraction from cyanobacteria (Dederich et al., 2002; Mehta, Evitt, & Swartz, 2015). (Dederich et al., 2002; Mehta et al., 2015). Using this principle, a protocol was established to verify if the glass beads were useful to improve the extractions from spores, with four different experimental conditions. Sample 1 was extracted only with the solvent, dichloromethane-methanol in the proportion of 2 to 1. (DCM:MeOH (2:1)). Secondly, in sample 2 the same solvent mixed with 40 glass beads with a diameter of 3 mm. Next, with sample 3, the spores were extracted with DCM:MeOH (2:1) with the help of sonicator (Q55 Sonicator). Finally, the sample 4 was submitted an extraction the solvent DCM:MeOH (2:1) with the glass beads plus sonication. All the samples were extracted in 3 cycles with a duration of 5 minutes of sonication each with 10 minutes of rest (in part to avoid the over-heating, and to give time to the solvent penetrate

in spores) in a plastic tube of 50ml. Particularly, the sample 2 and 4 were subjected to agitation with vortex, to force the mechanical lysis through the glass beads. At the final of each cycle, the supernatant was decanted to 4 plastics tubes. After all the cycles were completed, the samples were left to rest during 24h, and the supernatants were transfer to pre-weighted vials with the assistance of a Pasteur pipette. These vials were evaporated under reduced pressure, with a rotary evaporator (Buchi Rotavapor® R-100) and the recovered weight was calculated. The vials were weighed before and after placement of each crude extract in order to calculate the extraction yield. The weight of spores used in each condition and volume of solvent used are describe in the Tables 6 and 7. Giving the results obtained using only the glass beads, this procedure was repeated for the subsequent preparation of extracts.

3.4. Full scale extraction with glass beads method

Given the obtained higher performance of extraction with glass beads, they were chosen to perform mechanical lyses. Instead of performing a full extraction, it was decided to extract the spores with small quantities and volumes of solvent, in order to optimise the solid/solvent ratio. With an average of 800 mg per plastic tube, the spores were extracted with 30 ml of DCM:MeOH (2:1) in 2 cycles with 40 to 50 glass beads with 3 mm of diameter. During each cycle, the tubes were agitated with a vortex for 5 min, and then allowed 5 min to rest. At the end of the sequence, the supernatant was decanted to a glass flask. Totally, 61.29g of spores were extracted with a total volume of 2250 ml of DCM:MeOH (2:1) mixture. After 24h, the supernatant was retired to a glass balloon, carefully with the help of Pasteur pipettes not to perturb the deposit in the bottom of the flask. Next, the solvent was evaporated under reduced pressure, with a rotary evaporator and transferred with chloroform into pre-weight vial and evaporated again. This crude extract, with the code name E16152, was stored in a pre-weight vial, and the recovered mass and the respective yield calculated.

3.5. Fractionation of the E15094, PT01 and E16152 crude extract.

All crude extracts were fractioned through vacuum liquid chromatography method (VLC) (Targett, Kilcoyne, & Green, 1979). The 5g of silica gel (Merkel 0.015-0.040) were packed hard with support of vacuum and the crude extracted was transferred into the top of the column and 400ml of hexane were continually being added until the column absolved all

extract compounds. Continuing, a series of different mixtures with increasing polarity was charged in the column, until obtaining 9 distinctive fractions. (A with lowest polarity and I with highest). The mixtures of solvents and their volumes, along with each mass recovered from the fraction are described in the Tables 6, 7 and 8. The fractions were collected in round bottom flask and all the solvents were dried under reduced pressure, in a rotary evaporator. The dried fractions were dissolved in chloroform and transferred to pre-weight vials and evaporated again to determine the weight of the dried mass.

3.6. Sub-Fractionation of PTAFCES

The fractions with higher anticancer activity had such low masses, that isolating and testing the compound(s) responsible for that response would prove very difficult. Therefore, it was decided to collect all the fractions with higher activity from E15094, E16152, PT01 and E14030 (this extraction was performed by (Alves et al., 2015)) in a single collection called PTAFCES. Table 2 describes which fraction were pooled together and their mass.

Table 2. Composing fractions of PTAFCS and total mass obtained

Extraction code	Fractions	Total mass (mg)
E14030	G, H	133.43
E15094	F, G	
PT01	E, F, G	
E16152	E, F, G	

All the mass of the PTAFCES were sub-fractionated through a chromatography column. Here it was used 20g of silica gel (Merkel 0.015-0.040) as stationary phase and a variety of solvents mixtures with increasing polarity as mobile phase. In total 187 test tubes were collected in seven different elution mixtures (Table 3). Previously to this, Thin Layer Chromatography (TLC) was performed to establish the mobile phase that result in better separations of the compounds present in the extract. Later TLC was used to identify the tubes to mixture in the same pool. Under this technique, a small sample (10 to 20 μ L) of the fraction was collected from every 5 tubes with a capillary, and put in a TLC plate. The same plate was placed in a TLC chamber with the mobile phase with increasing polarity,

to drag the most polar substances. All the spots present were verified under a UV light and marked with a pencil.

Table 3. Mixing ratio, volume and type of solvents used to perform the gravitational chromatography from PTAFCES

Tubes	Solvent	Mixing ratio (%)	Volume (mL)
1-46	Hex- EtOAc	60-40	300
47-62	Hex- EtOAc	50-50	100
63-106	Hex- EtOAc	40-60	300
107-127	Hex- EtOAc	30-70	100
123-138	Hex- EtOAc	20-80	100
139-155	Hex- EtOAc	10-80	100
156-171	EtOAc	100	100
172-187	EtOAc-MeOH	50-50	100

It was analysed the spots marked on the TLC and regroup the tubes solutions by the presence of the same, or very similar compounds. This reorganization outcome in 22 fractions, resulting of being transferred to pre-weighted vials, evaporated under reduced pressure, and calculated the recovered mass. (Table 4)

Table 4. Fraction obtained with each tube and total mass recovered from PTAFCES chromatography

Fractions	Tubes	Mass (mg)	Total mass recovered (mg)
1	1-5	0.76	105.35
2	6-8	2.31	
3	9-11	1.01	
4	12-17	2.42	
5	19-30	26.83	
6	31-39	8.56	
7	40-46	6.84	
8	47-50	2.9	
9	51-62	8.51	
10	63-67	3.06	
11	68-76	4.10	
12	77-89	2.29	
13	90-101	2.03	
14	102-124	2.71	

15	125-133	1.27
16	134-142	1.14
17	143-154	1.22
18	155-168	1.26
19	169-175	0.51
20	176-179	10.09
21	120-181	7.41
22	182-187	8.07

3.7. Preparation of the test samples

From the crude extractions and the fractions E15094, PT01 and E16152 a test sample at a concentration of 100mg/ml were prepared. The dried weight was dissolved in chloroform in a vial, and from this solution successive dilutions were done in order to prepare other concentration test of 10mg/ml and 0.1mg/ml. These vials were evaporated using a nitrogen stream and 100 μ l of dimethyl sulfoxide (DMSO) were added to each vial. The fractions from the collection PTAFCES were under the same process, with the exception that a unique concentration of 0.2mg/ml was prepared.

3.8. Preparation of the PTAFCES for nuclear magnetic resonance spectroscopy (NMR)

To verify the purity and to evaluate the chromatography efficacy observe the chemical structure of the compounds present in each sample, the 22 fractions from PTAFCES were submitted to ^1H NMR analysis. Each NMR tube was pre-washed with acetone, chloroform and methanol to eliminate the presence of contaminants and dried thoroughly in high vacuum. Each fraction was solubilized in deuteriochloroform and loaded into the prepared NMR tubes. Each fraction was solubilized in deuteriochloroform (CDCl_3) and loaded into the prepared NMR tubes. The ^1H NMR spectroscopy was carried out at Centro de Materiais da Universidade do Porto (CEMUP), using a BRUKER AVANCE III 400 MHz, 9.4 Tesla to perform this assay.

3.9. Chromatographic scan using High Pressure Liquid Chromatography (HPLC) of PTAFCES samples

In the results from the cytotoxic essay, revealed a strong cytotoxic activity in the fractions 12-19. When these results were compared with NMR spectra, it was possible to identify some common peaks, showing the identical compounds are present in different fractions. Given this, the PTAFCES 12-19 was submitted to High Pressure Liquid Chromatography (HPLC) to verify the chromatography profile of this set of fractions. The 10 μ l of PTAFCES 12-19 was injected in a Synergi 4u Fusion-RP 80A 250x10mm column, with a flow of 2.0ml/min, with absorbance read at 280 nm and 254 nm, with an isocratic mixture of 100% MeCN. It is important to note it was only injected the soluble part in MeCN, with other part being preserved.

3.10. Cell Cultures

The cytotoxicity of samples was tested against 3 cancer cell lines and 1 normal cell line:

- T47D (human breast cancer cell line);
- RKO (human colon adenocarcinoma cell);
- Hep G2 (hepatocyte carcinoma);
- hCMEC/D3. (human brain capillary endothelial cells)

Cells were incubated at 37°C with a 5% CO₂ humidified atmosphere, and culture medium was renewed every 2 days. When flask fill up with 80% cell confluence, adherent cells were free with TrypLE Express Enzyme (Gibco and culture medium was renewed every 2 days. Considering this, the T47D cells were purchased from Sigma-Aldrich, RKO and Hep G2 cell lines were obtained from the American Type Culture Collection (ATCC) and finally the hCMEC/D3 were provided by Dr. P. O. Couraud (INSERM,France). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, GlutaMAX, Life Technologies), pH 7.4, supplemented with 10% fetal bovine serum, 2.5 μ g/ml fungizone, and penicillin–streptomycin (100 IU/ml and 100 μ g/ml, respectively).

3.11. Cell viability assay

The (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay is a colorimetric test to measure mitochondrial activity. In this context, the mitochondria's are responsible to reduce this yellow dye into formazan purple crystals and so, the absorbance of this colored solution can be read by a microplate reader (van Meerloo, Kaspers, & Cloos, 2011). This mitochondrial activity can be proportional to cell viability considering the 100% viability in the control (cells treated with 1% DMSO). For the cell viability assay, the cells were seeded with a density of $3,3 \times 10^4$ cel/ml in triplicate in 96 well plates (previously counted in a Neubauer chamber). After 24h of incubation, the medium was removed and 1 μ l of the fractions samples diluted in 100 μ l of fresh medium was inserted, to cells being expose to 100, 10 and 1 μ l/ml (the PTAFCES samples were tested at 20 μ l/ml) during 24h, 48h and 72h. Furthermore, were establish positive controls (cell being exposure with medium and 1% of DMSO) and negative controls (cells treated with medium and 20% of DMSO). After this period of exposure, the cells were treated with 10 μ l of MTT at a concentration of 0.05mg/ml with an incubation period of 4h. After this time, the cell medium was retired and 100 μ l of DMSO were added to solubilize the purple formazine salts. The absorbance of these crystals was measure with microplate reader (Bio-tek Synergy™ HT) at of 550 nm, and results expressed as a percentage of cell viability considering 100% viability in the control cells.

3.12. Statistical Analysis

Microsoft Office Excel 2016 was used to treat the data from MTT assay. The results are presented as means \pm standard deviation (SD) from three independent experiments, when compared with control results. The same software was used to display the data graphically.

4. Results

4.1. Improving the extraction technique

Improvement of the efficiency of the extraction was crucial to obtain sufficient mass to use a bioactivity assay guided isolation of compounds (using anticancer assays) and to perform the characterization of the same substances. The four-different conditions of extractions resulted in distinct extraction yields. Additionally, all the experimental conditions used the same volume of solvent mixture, submitted to the same time cycles, (with some slight differences in the weight of spore's due to the scale sensitivity). Giving this, the standard extraction (sample 3) previously used by Alves et al., 2015 had resulted in 7.50 % of efficiency, being the lowest yield among the four, contrasting with the highest obtained with 40 glass beads plus sonication (17.71%). The use of only solvent to perform the extraction had given a yield of 8.61% and the use of the 40 glass beads resulted in an yield of 16.5%. Ideally, sample 4 would show the highest efficiency extraction and logical the first choice to be use in the final extraction, however the method used with sample 2 was chosen to perform the large-scale extraction.

Table 5. The four-different conditions of experimental extractions and respective recovered mass and efficiency yield

Sample	Condition	Cycles and time	Solvent volume (ml)	Solvent	Spores weight (mg)	Weight after the extraction (mg)	Extraction Yield (%)
1	Only solvent	3 cycles; 5 min each	20 ml each cycle; 60 ml in total	DCM:MeOH (2:1)	109.9	7.9	8.61

2	40 glass beads	3 cycles; 5 min each with agitation by vortex	20 ml each cycle; 60 ml in total	DCM:MeOH (2:1)	97.2	16.1	16.5
3	Sonication	3 cycles; 5 min submitted in sonication	20 ml each cycle; 60 ml in total	DCM:MeOH (2:1)	96.2	7.8	7.50
4	40 glass beads; Sonication	3 cycles; 5 min with agitation by vortex; 2 min submitted to sonication	20 ml each cycle; 60 ml in total	DCM:MeOH (2:1)	112	15.8	17.71

4.2. Fractionation of the E15094, PT01 and E16152 crude extract

Following the extraction method of Alves et al. (2015) it was possible to obtain two crude extracts, as cited above, with the codenames of E15094 and PT01, with yields of 1.9% and 2.2%, respectively. After a detail observation of the fractionation table data of E15094 (Table 6), it is possible to verify that 1.9% of the biomass was recovered with the standard protocol by Alves et al., 2015. Using this polarity gradient and looking to the results from Alves et al., 2015 this VLC was performed to obtain nine fractions with different weights between them. Fraction A and I represented more than half of the total weight, with 23.5% and 32.4%, respectively. Contrasting these results the fractions B (10.0 mg) and C (12.2 mg) denoted the lowest weight fractions.

Table 6. Data about E15094 fractionation through VLC and extraction procedure

Extract name	Spores weight (g)	Recovered mass (mg) and yield extraction (%)	Fractions	Solvent	Mixing ratio (%)	Volume (mL)	Fractions mass (mg)
E15094	50.0	950; 1.9%	A	Hex-EtOAc	90-10	200	223.6
			B	Hex-EtOAc	80-20	100	10.0
			C	Hex-EtOAc	60-40	100	12.2
			D	Hex-EtOAc	50-50	100	34.3
			E	Hex-EtOAc	40-60	100	127.6
			F	Hex-EtOAc	20-80	100	104.5
			G	EtOAc	100	150	40.0
			H	EtOAc-MeOH	75-25	150	89.2
			I	MeOH	100	300	308.1

Results from the extraction and respective fractionation of PT01 (Table 7) revealed a light increase of the yield by 0.3 % when compared with the E15094. This same extract was divided in nine fractions, following the same weight pattern, with the fraction B and I having most mass of the extract. Furthermore, the fractions D, E, F and G only totaling 13.35 mg, represent 1.3% of the total mass of the extract. The use of glass beads in the extraction process, almost duplicated the yield when compared with the previous extracts. With an efficiency of 4.0%, it was possible to recover 2388mg of extract, distributed in 9 fractions. After a more detailed observation of Tables 6, 7 and 8, it is possible to affirm, that the fractions A and I are the ones with more mass, comparing with F and G, which are the fraction that contain less mass. Nevertheless, these fractions have more mass when comparing with the ones from PT01.

Table 7. Data about PT01 fractionation through VLC and extraction procedure

Extract name	Spores weight (g)	Recovered mass (mg) and yield extraction (%)	Fractions	Solvent	Mixing ratio (%)	Volume (mL)	Fractions mass (mg)
PT01	44.42	977mg 2.2%	A	Hex-EtOAc	90-10	400	139.93
			B	Hex-EtOAc	80-20	250	202.53
			C	Hex-EtOAc	60-40	250	12.46
			D	Hex-EtOAc	50-50	250	3.16
			E	Hex-EtOAc	40-60	250	4.95
			F	Hex-EtOAc	20-80	250	3.01
			G	EtOAc	100	250	2.23
			H	EtOAc-MeOH	75-25	250	47.81
			I	MeOH	100	500	597.11

When compared with the glass beads extraction E16152 (2388mg) with a yield of 4.0%, the recovered weight of the E15094 and PT01 (950mg and 977mg) was considered very low, proving that this method improves the spore's extractions in large-scale extractions.

Table 8. Data about E16152 fractionation through VLC procedure

Extract name	Spores weight (g)	Recovered mass (mg) and yield extraction (%)	Fractions	Solvent	Mixing ratio (%)	Volume (mL)	Fractions mass (mg)
E16152	61.29	2388mg 4.0%	A	Hex-EtOAc	90-10	400	427.60
			B	Hex-EtOAc	80-20	300	159.94
			C	Hex-EtOAc	60-40	250	220.61
			D	Hex-EtOAc	50-50	250	129.9
			E	Hex-EtOAc	40-60	250	60.66
			F	Hex-EtOAc	20-80	250	25.47
			G	EtOAc	100	250	15.51
			H	EtOAc-MeOH	75-25	250	82.0
			I	MeOH	100	600	642.38

4.3. The NMR spectra from the PTAFCES.

The NMR analyses from PTAFCES fractions showed multiple peaks differing between the 22 fractions. Therefore, the analyses (Figure 4) revealed that the fractions still complex, with the presence of multiple compounds. However, some common peaks were found, generally between the fractions 12-19, with some variance in the strength of the signal. It was possible to observe the presences of doublets at 7.90 δ , 7.59 δ and quartet 7.10 δ . Additionally, in all PTAFCES NMR spectra it is possible to observe strong signals, with singlets at 7.26 δ and 3.49 δ

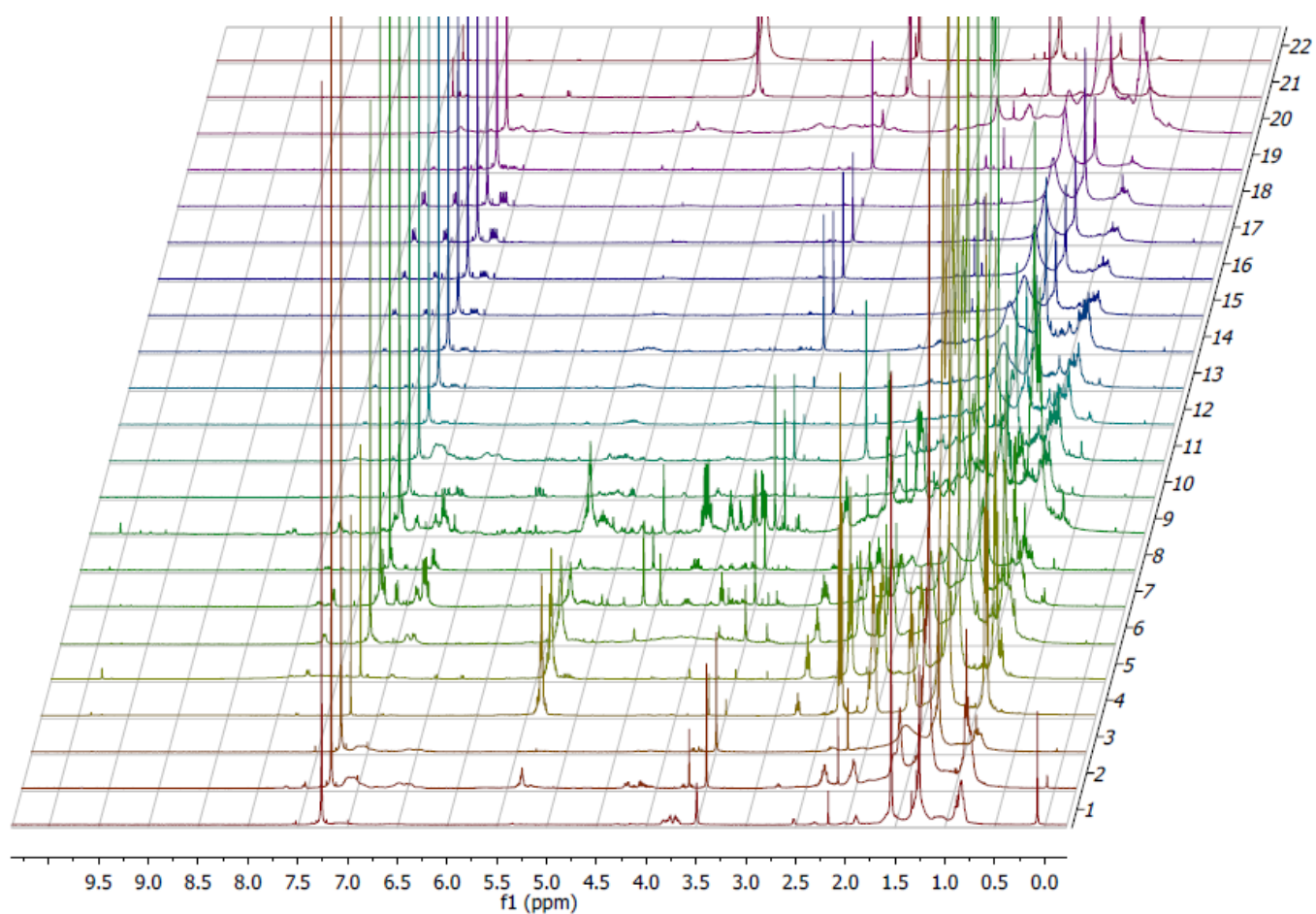


Figure 4. NMR spectrum from the 22 fractions of PTAFCES fractions.

4.4. Results from the chromatograph scan

Figure 16 presents the chromatograph profile of the extraction collection PTAFCES, with the blue and the red line representing the absorbance at 280 nm and 254 nm, respectively. In the chromatograph is possible to verify the presence of numerous peaks in the two wavelengths, in specific between the 6:00min and 23:00min. For instance, the first detected peak had a one strong signal, noticed at 6:30 min, corresponding to the solvent injection peak.

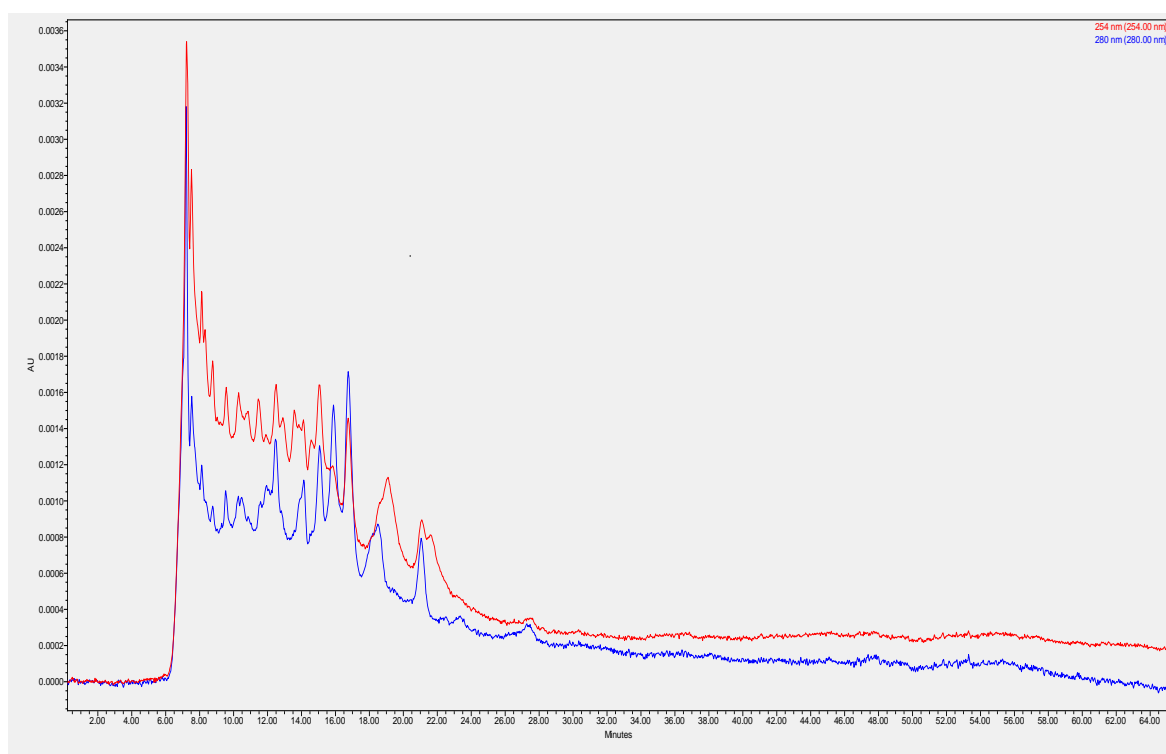


Figure 16. Chromatographic profile of the PTAFCES 12-19 pool. The blue and the red line represents the absorbance at 280 nm and 254nm respectively.

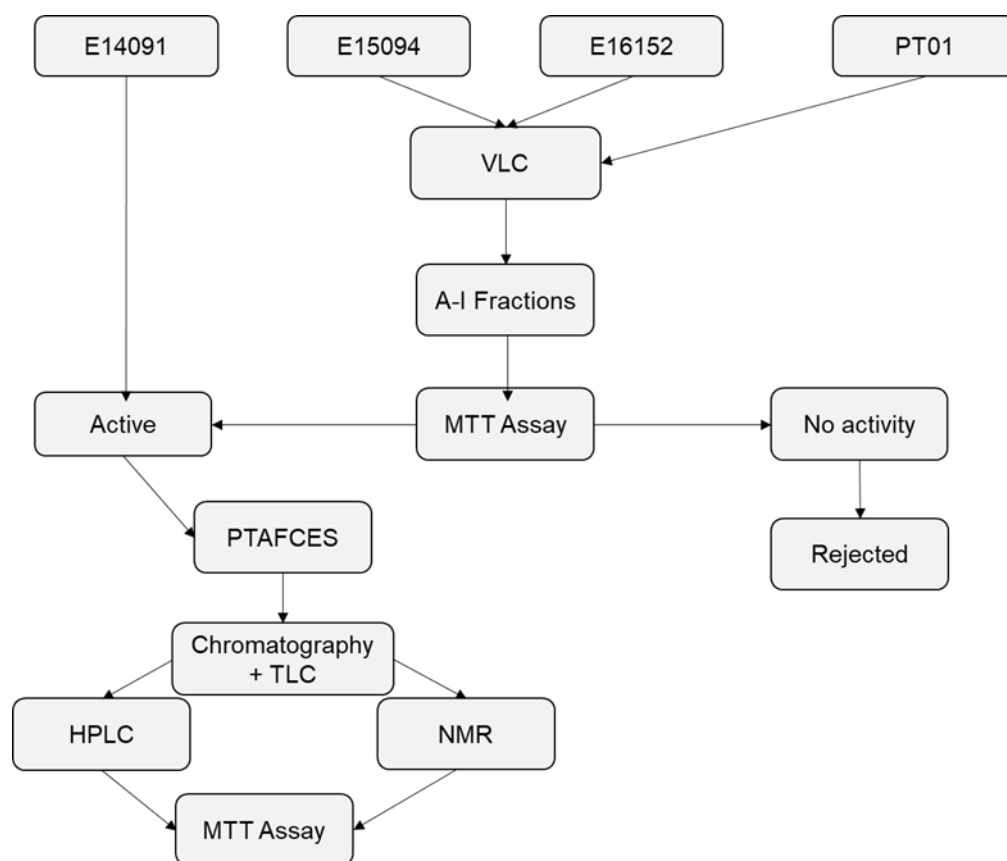


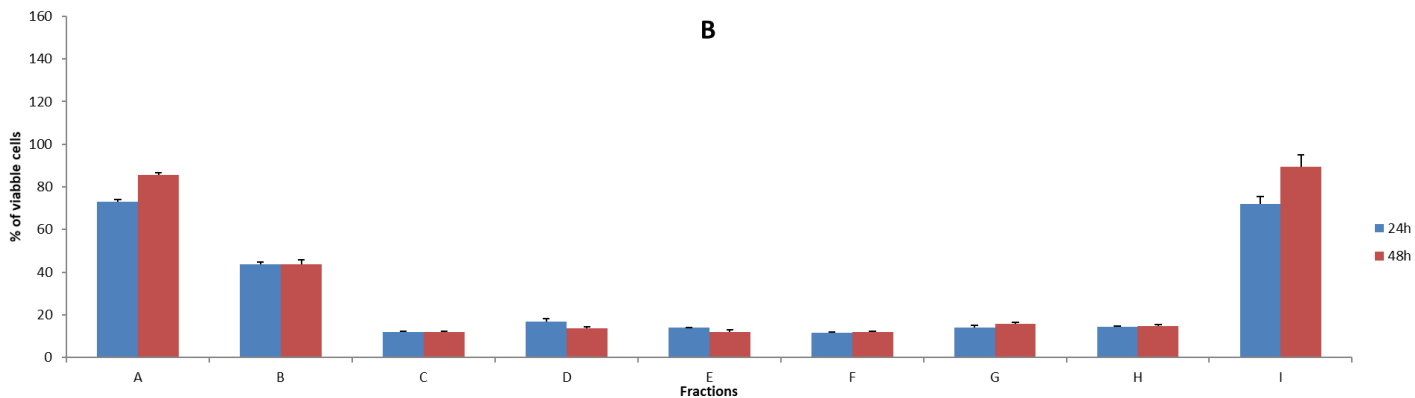
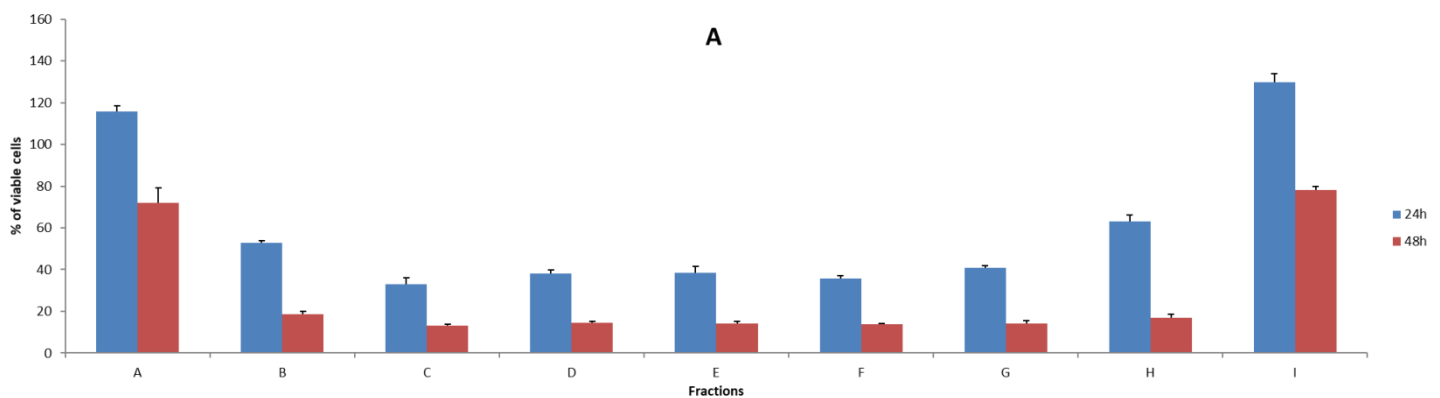
Figure 5. Bioassay guided fractionation design used in this study

4.5. Cell viability results

4.5.1. Cell viability results from E15094

The E15094 fractions were tested for cell viability at a concentration of 10µg/ml with time of exposure of 24h and 48h. Giving this, the figure 8 shows the effects of the fractions in the in the cancer and HCMEC/D3 cell viability. Regarding the T47D cell viability, the fraction A and I are the less toxic with 86% of the cells being viable after 48h. In other hand, only the 10% and 12% of the cells were viable when treated with the fraction F and G during 48h. The exposure of the RKO cell lines to the fraction A and I manifest less toxicity, with 73% and 74% of viability after 24h, with a slight increase 85% and 89% after

48h, respectively. Moreover, the fractions E and F demonstrate the strongest decrease of the cell viability (12% and 11% after 48h), with no relevant differences between the time exposure. Following the same pattern of the results obtained in HCMEC/D3. The data demonstrate that fractions A and I exhibit an increase of the number of the viable cells, superior to the control group in 120% after 24h of exposure, then dropping to 72% and 78% after an exposure of 48h, being the ones with less impact in the viability of the HCMEC/D3 cells. On contrary, the exposure of the fractions E and F result in only 38% and 37% of the cells were viable after 48h. In fact, by prolonging for 48h, the results are more acute with the viability being 13% and 14%, respectively.



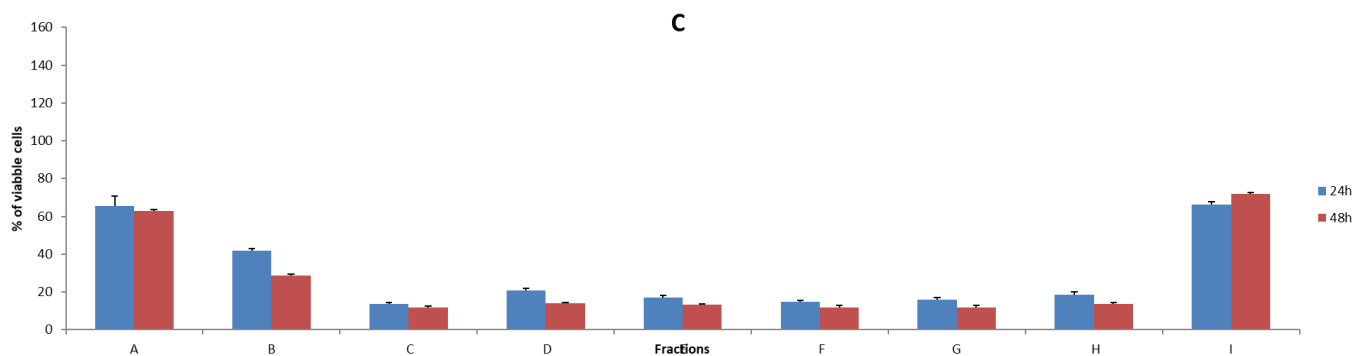
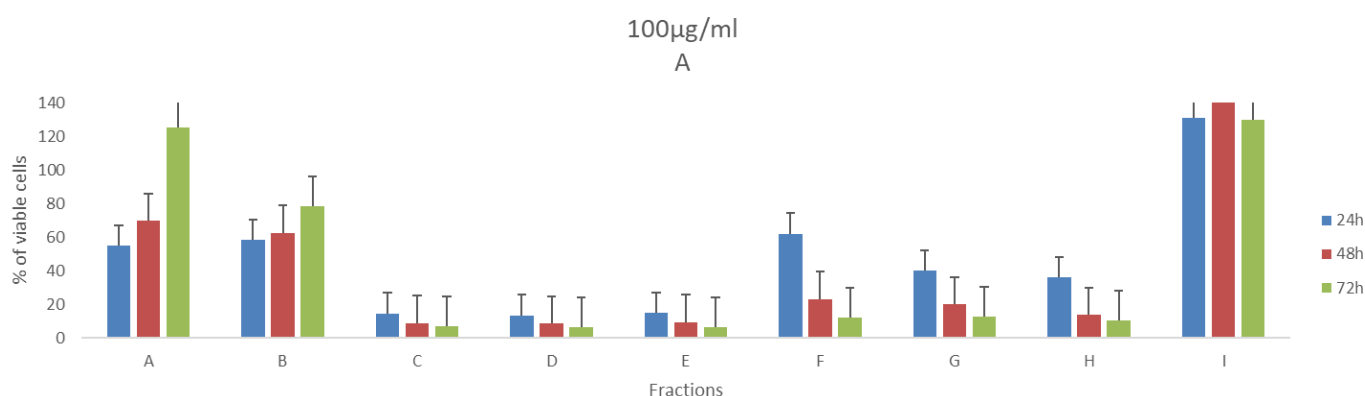


Figure 6. Effects of fractions from E15094 in 10µg/ml in the viability of HDMC (A), RKO (B) and T47D (C) Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

4.5.2. Cell viability results from PT01

The figures 7-10 illustrates the cell viability from exposure of nine fractions (in 100µg/ml, 10µg/ml and 1 µg/ml) from PT01 in four cell lines, during three-time periods. Concerning the effects of the fractions on viability of the T47D cells (Figure 7), it is clearly visible that the highest concentration provokes the lowest number of viable cells after 72h, more specifically the fractions C, D, E, F, and G. Yet, fractions E, F and G can still reduce drastically the viability the cell when exposed to the lowest concentration (1µg/ml) to 12% at 72h. It is also important to note, that in fraction I it was noticed an increase of the number of viable cells when compared with the control in all concentrations, more precisely 74% at 1µg/ml. It is also interesting results from fraction B, show to be more active in lowest concentration than other highest two.



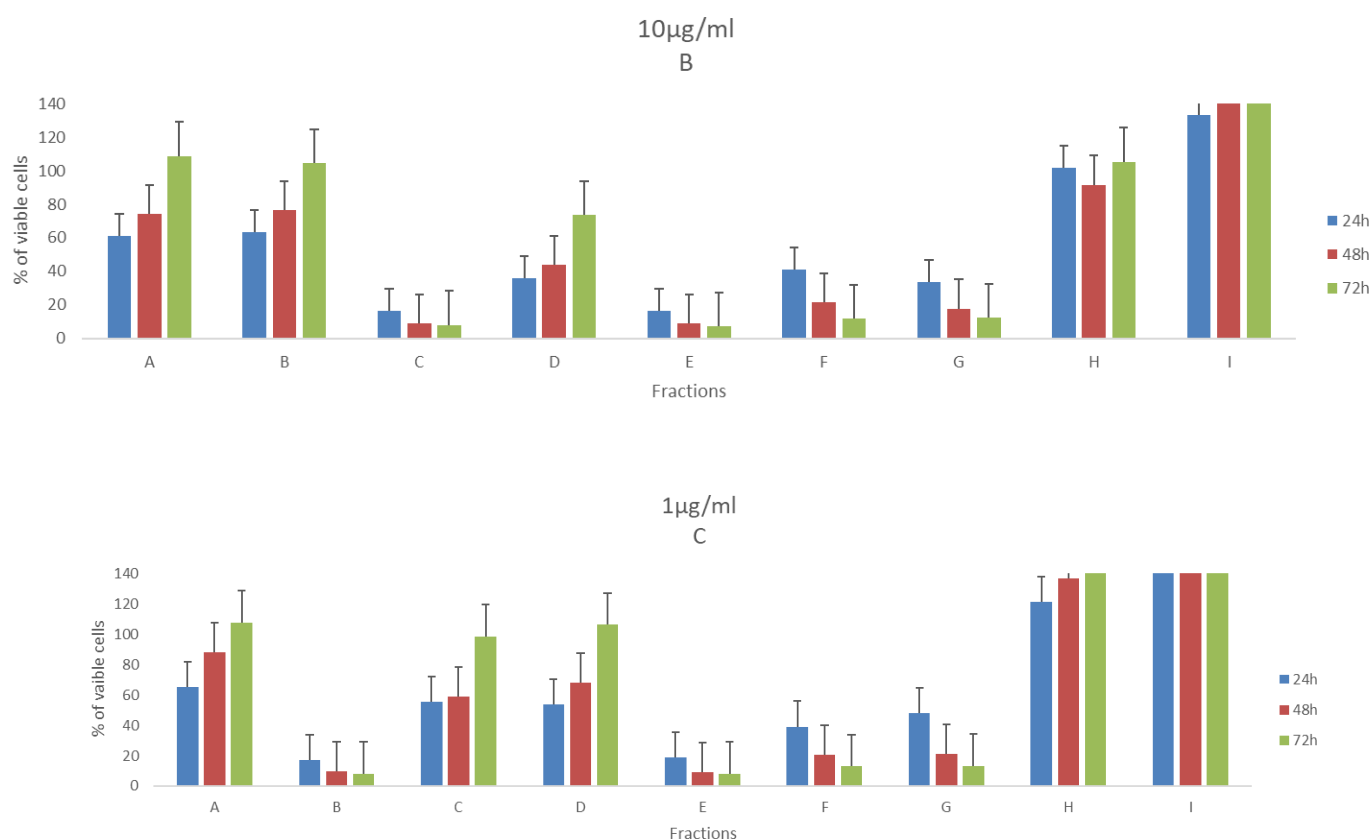


Figure 7. Effects of fractions from PT01 in 100µg/ml (A), 10µg/ml (B) and 1µg/ml (C) in the viability of T47D. Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

Regarding the RKO cell lines (figure 8), the most active fractions are the C, (the Fraction C in 1µg/ml showed low impact on the cell viability) D, E, F and G at 72h. On the contrary, it is possible to observe a cellular proliferation, when treated with the fractions A and I and compared with control, displaying no signs of being toxic at that concentration test.

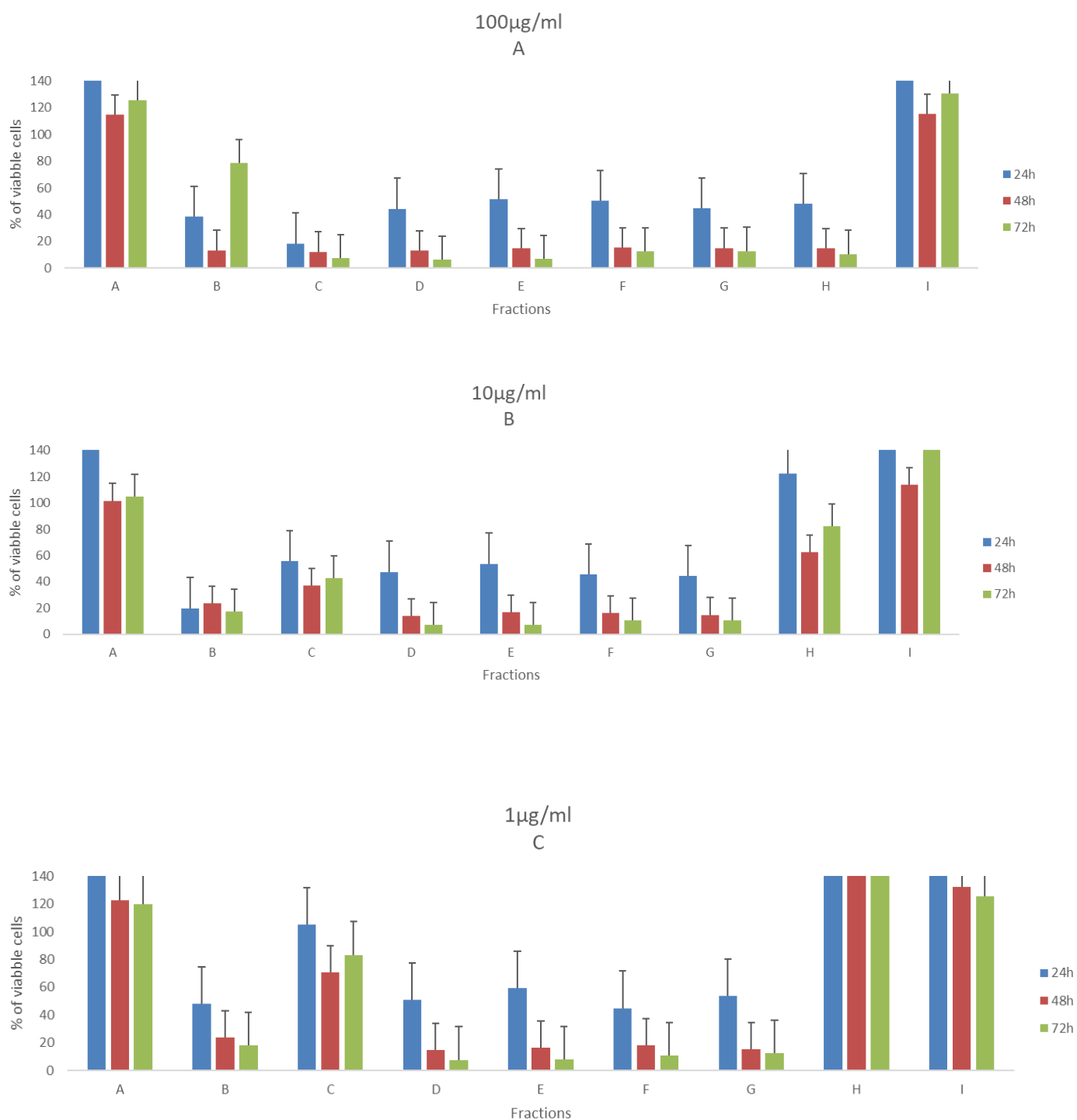


Figure 8. Effects of fractions from PT01 in 100µg/ml (A), 10µg/ml (B) and 1µg/ml (C) in the viability of RKO. Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

Next, the fractions E, F and G prove to reduce the cell viability significantly in the HEPG2 even in the lowest concentration, with only 6%, 12% and 14% viable cells, respectively

after 72h (Figure 9). As like the other cancer cells, the fraction I stimulated the growth of HEPG2 cells, when tested at a concentration of 100µg/ml and 10µg/ml.

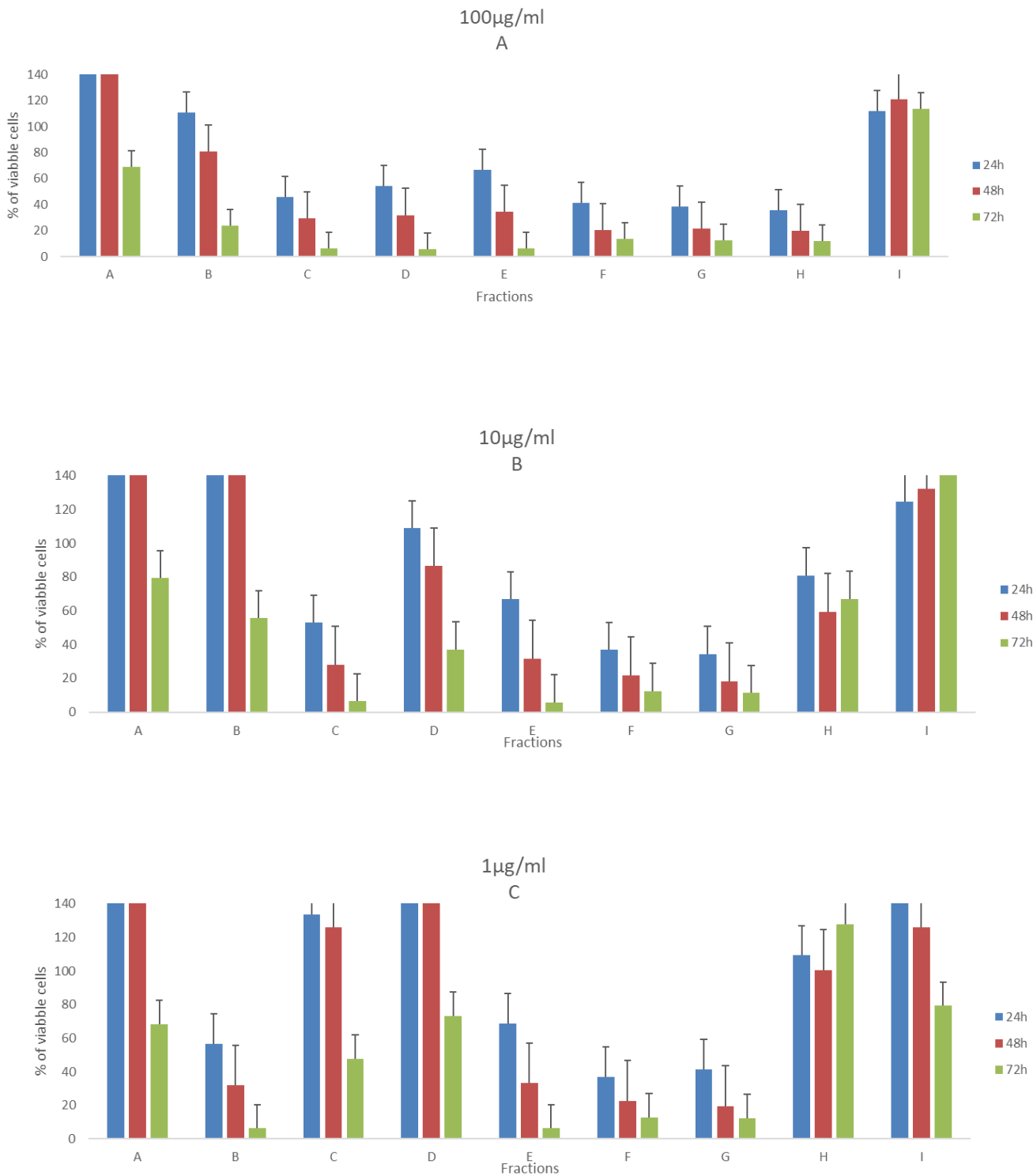
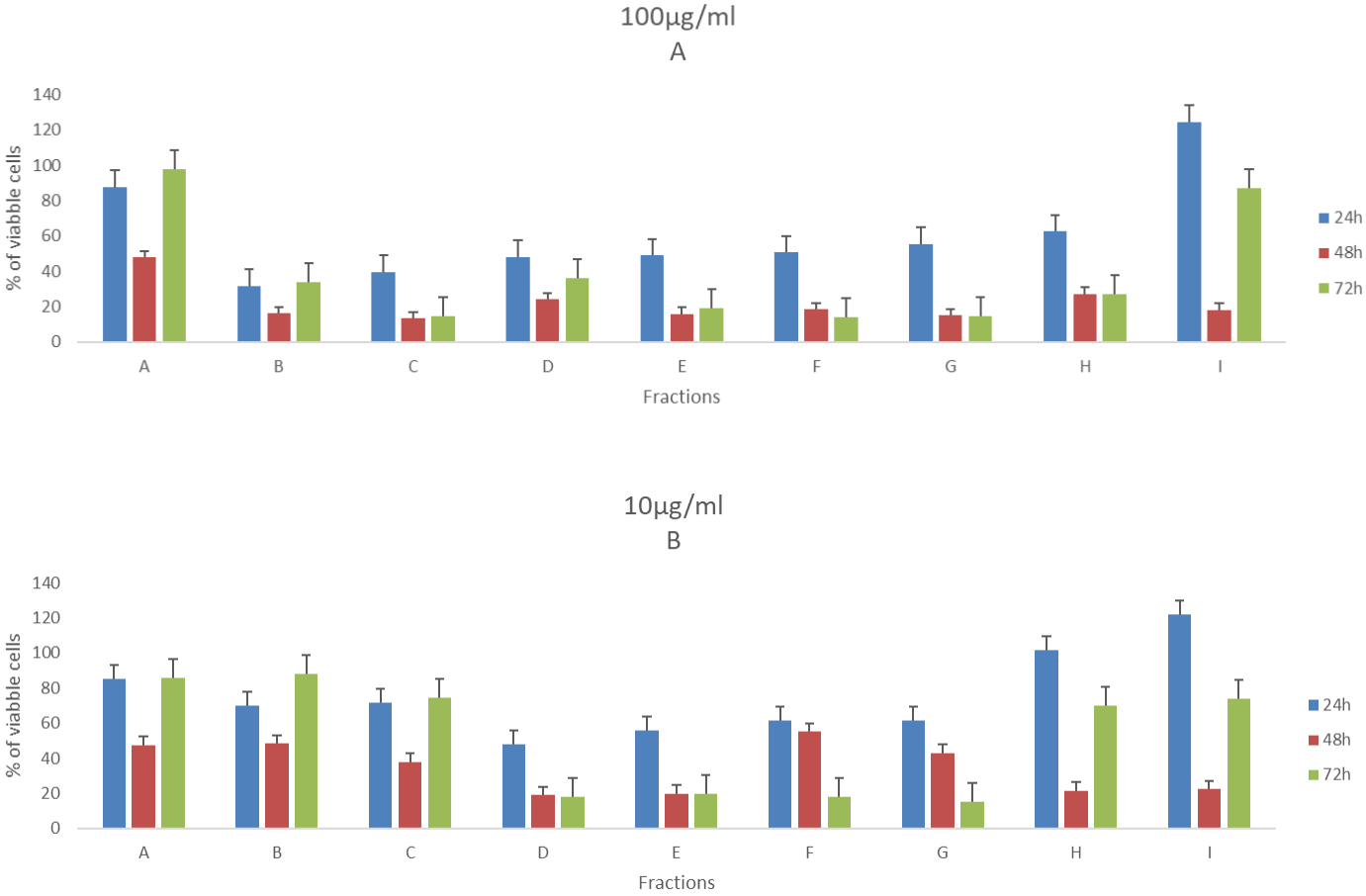


Figure 9. Effects of fractions from PT01 in 100µg/ml (A), 10µg/ml (B) and 1µg/ml (C) in the viability of HEPG2. Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

Finally, the HCMEC/D3 cells (Figure 10) were particular affected by the exposure to all tested concentrations. Moreover, the fraction F showed to be the most toxic in all the concentrations tested after 72h of treatment. The data revealed also a non-time dependent response, with the cellular viability being specifically severe at 42h, increasing steadily in the 72h period.



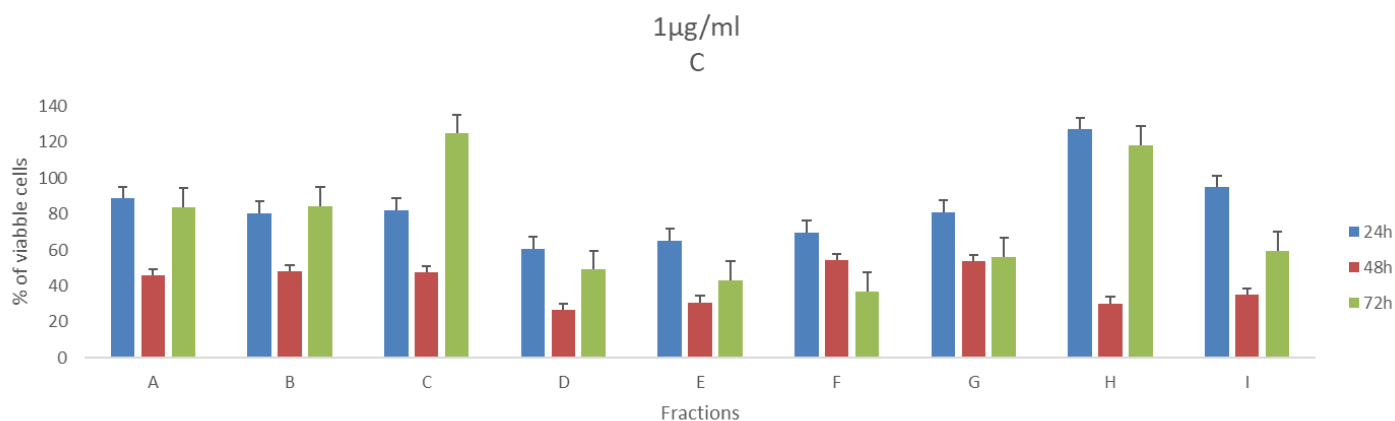


Figure 10. Effects of fractions from PT01 in 100µg/ml (A), 10µg/ml (B) and 1µg/ml (C) in the viability of HDMC. Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

4.5.3. Cell viability results from E16151

The figure 11-14 exhibits the cellular viability from four diverse cell lines, during three-time periods, when exposed to three different test concentrations of nine fractions of E16151 extract.

Concerning this assay with T47D cell lines (Figure 11), the fractions C, D, E, F, G, H and I showed a pronounced toxicity when treated with 100µg/ml after 72 h. Moreover, the fraction E proved to be the one to cause less cellular viability (12%). The same pattern repeats when exposure to 10µg/ml, except for the H and I, that exhibits a cellular proliferation when compared with the control group. At the lower concentration, most of the test fractions stimulated the cellular viability, excluding the fractions E, F and G, with a cell viability of 54%, 77% and 55% after 72h, respectively.

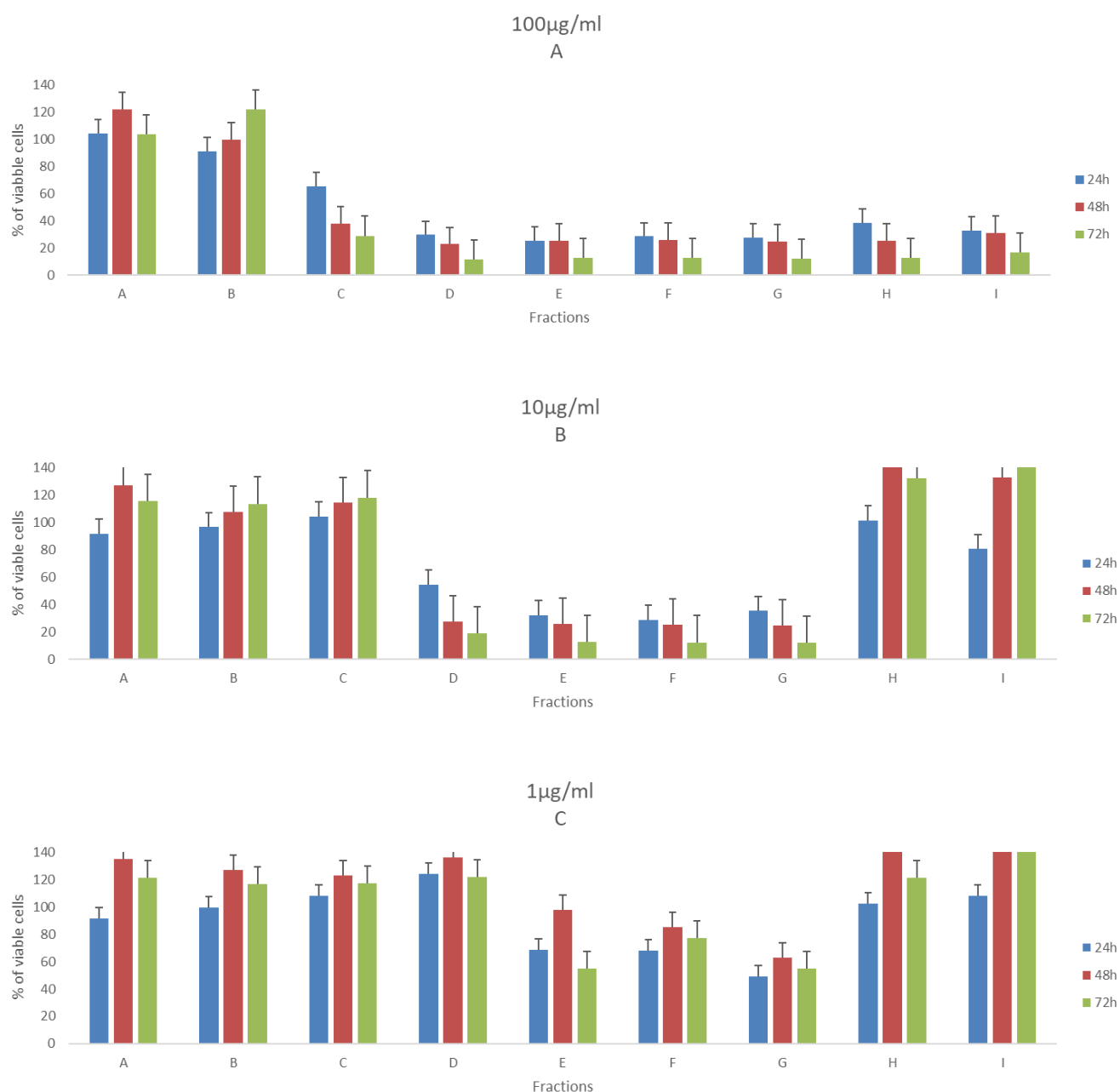


Figure 11 -Effects of fractions from E16151 in 100µg/ml (A), 10µg/ml (B) and 1µg/ml (C) in the viability of T47D. Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

The data demonstrates that the results from RKO are similar to T47D (Figure 12). The most active fractions are C, D, E, F, G, H and I after 72h in the higher test concentration. The viability had a vigorous increase in the fraction H and I, being observed stimulation of the number of viable cells, when exposed to 10µg/ml. Although, the RKO cells when treated with 1µg/ml, showed high cell viability, with the most the fraction G, the most active

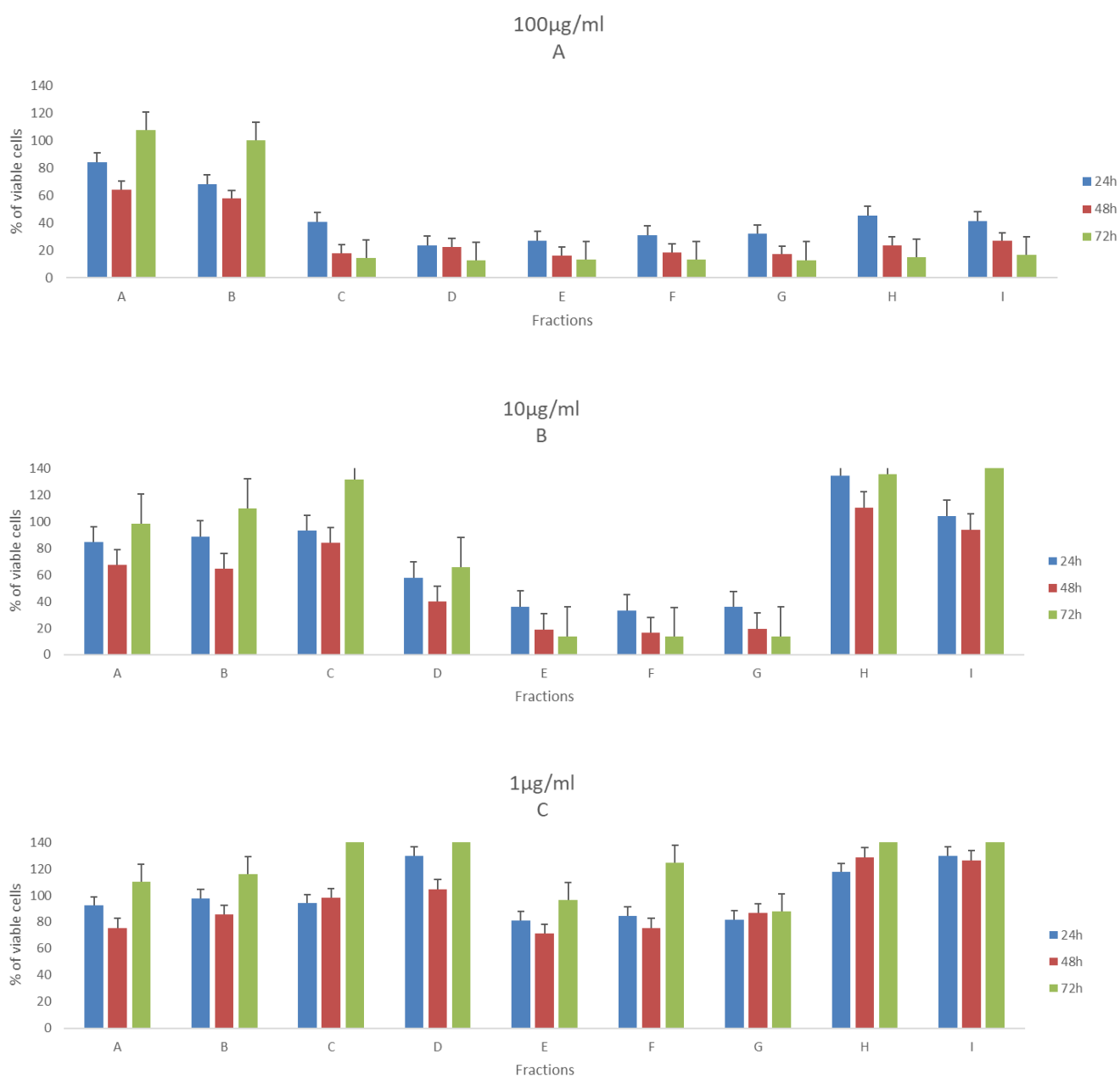


Figure 12. Effects of fractions from E16151 in 100µg/ml (A), 10µg/ml (B) and 1µg/ml (C) in the viability of RKO. Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

Following the HEPG2 cell lines, (Figure 13) the most active fraction were E, F and G with only 5%, 6% and 5%, respectively were viable after 72h in the highest concentration. As expected, when the cells are treated with 1µg/ml, show a higher viability when compared with other higher concentrations, being the fraction E the most toxic for the cells (23%). On this data it is clearly perceptible, a time-dependence for effects on the cellular viability. In fact, the toxic effects are more pronounced as the time of exposure is longer.

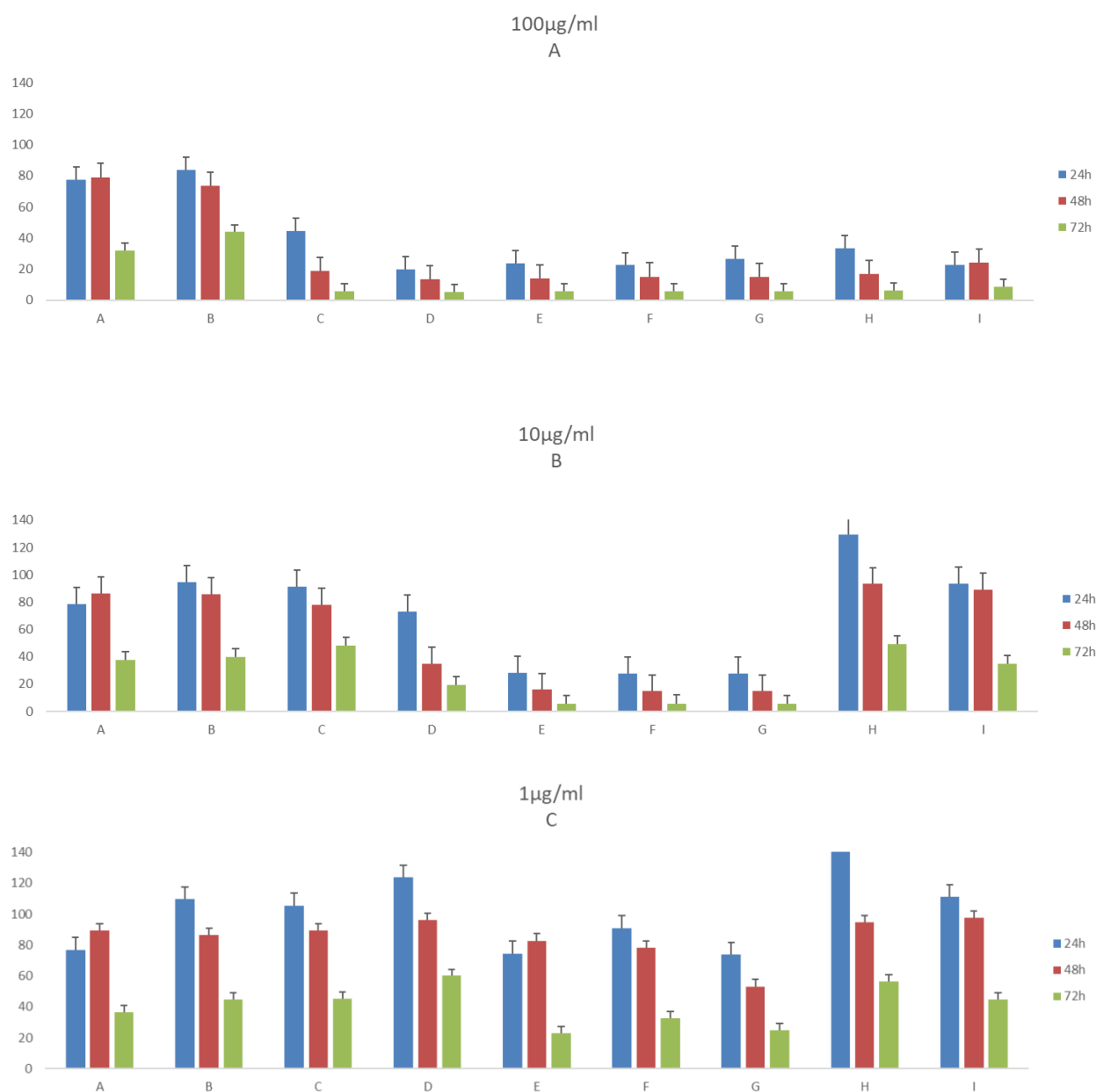


Figure 13. Effects of fractions from E16151 in 100µg/ml (A), 10µg/ml (B) and 1µg/ml (C) in the viability of HEPG2. Values are present as means ±SD of triplicate analyses when compared with control group (n = 3).

Finally, the data relative of the HCMEC/D3 (Figure 14) cell viability exhibits time-dependence response in all fractions (with exception of fraction I when treated with 10 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$). In the concentration of 100 $\mu\text{g/ml}$, the fraction D were the most active fraction (17%), contrasting with the test concentration of 10 $\mu\text{g/ml}$ where the fraction D cause the lowest number of viable cells after 74 h. It is also important to state that no toxicity was verified after being exposure to all concentrations in a time of 24 h.

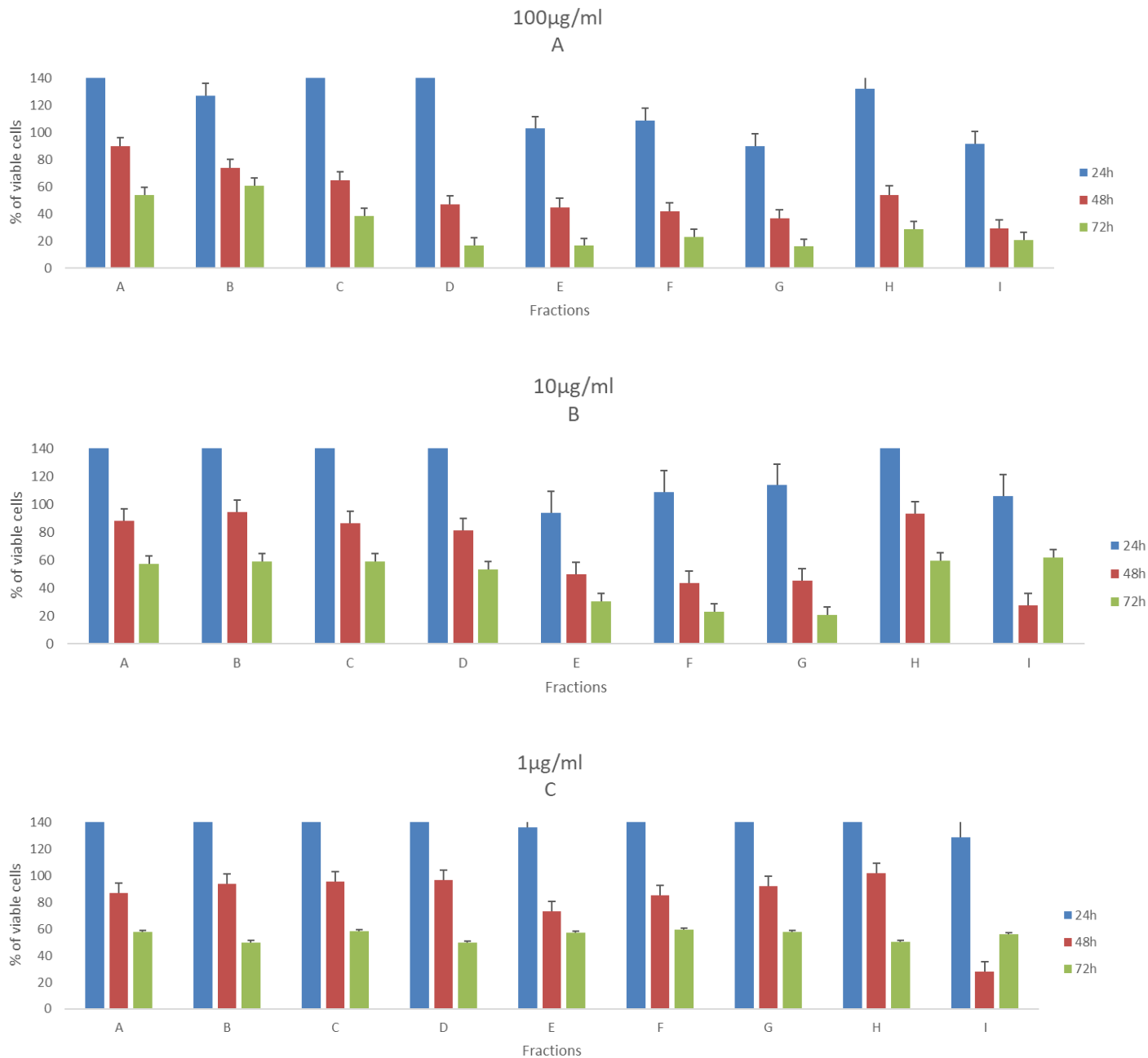


Figure 14. Effects of fractions from E16151 in 100 $\mu\text{g/ml}$ (A), 10 $\mu\text{g/ml}$ (B) and 1 $\mu\text{g/ml}$ (C) in the viability of HDMC. Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

4.5.4. Cell viability results from PTAFCES

The figure 15 illustrates the data on the cellular viability in two cell lines (HEPG2 and HCMEC/D3) when exposed to the 22 fractions that compose the PTAFCES extract collection at 20 µg/ml concentration. The fractions 4 to 18 caused low cellular viability in HEPG2 line, being the fraction 11 the most toxic after 72 h (9% of the cells were viable). This group of fractions showed a time-dependence response, since the increasing time of exposure, reduce the cellular viability. On the other hand, the fraction 21 and 22 exhibited no toxicity and even induced cellular proliferation.

Regarding the HCMEC/D3 cell lines, the fractions 4, 5, 6, 7, 9, 10, 11, 12, 13, 15, 16 and 17 provoked noticeable low cell viabilities after 72 h, with a typical response of a time-dependence. Moreover, observing the data about the cell viability it is possible to affirm that fraction 9 was the most toxic of the 22 tested fractions. Regardless of this, in fractions 8 the number of viable cells are the same as the group control, reporting no toxicity in all the three-time periods. Finally, the half the cells that were tested with fraction 9 were viable, after 24 h, 48 h and 72 h.

5. Discussion

Alves et al., 2015 were the first in the literature reporting the potential presence of bioactive compounds in *P. tinctorius* spores and given the lack of data, it was essential to establish an extraction method with better efficiency. In this work, an extraction yield of 2.94%, was obtained, which was considered low, considering the small masses of the active fractions obtained. As new in the present work was the use of small glass (or zircon) beads to break tick structures such as cyanobacteria cellular membrane (Mehta et al., 2015), disrupt bacteria spores (Altmeyer et al., 2012) or even to perform DNA extractions (Dederich et al., 2002). E15095 and PT01 were performed according to Alves et al., 2015, resulting in very low yield. Associate with this was the fact that most active fractions, were the ones with lower masses. (with the example of the fraction G from E15094 representing just 1.92% of total mass of the extract), it was imperative to optimize the extraction process.

So, observing the four different extractions (table 5), the sample 4, should be selected for the large-scale extractions for having the highest yield extraction. However, the use of the glass beads with sonication, with small mass and volumes (in order to optimize the surface/solvent ratio) would not prove to be time efficient, consequently resulting in logistical problems. Therefore, the difference between this method extraction and the one with only glass beads was consider negligible.

The results from extraction performed in small scales, are generally more efficient than the ones made in large scales. Taking into account the experience to choose the best extraction solvent, Alves et al., 2015 obtained a yield of 16.3% in a small scale, when in the final extraction only 2.94% of the initial mass was recovered. In this study, the same reduction of efficiency was verified. In the large scale, the extraction rate was reduced by 75% when compared with small scale, resulting in a efficiency of 4%. The increase of the ratio surface/volume in the extraction method could be an explanation of the low efficiency of the process. Maintaining this ratio and performing this extraction with 61.29g of spores it would be necessary to use 33.43L of the solvent mixture of DCM:MeOH (2:1). Using this volume, it would prove to be an economical and logistical issue, as well as handling such amounts could result in chemical hazard and jeopardize the laboratorial safety. The extraction of the E16152 to be proved be more efficient by increasing 2.1% and 1.8% when compared with ones performed in the E15094 and PT01, respectively. This

represents a step in the improvement of the process and confirms that this method can be used as baseline in other future extractions. Yet, this technique could achieve more improvements to increase more the extraction yield. Extraction of more biomass combined with the reduction the ratio surface/volume would prove good options to optimize the process. The 3 mm glass beads used in this extraction, were the ones also applied to force the lyses in cyanobacteria's. Vandeventer et al., 2011 stated that the diameter of the beads is crucial for increasing the yield extraction and 100 μm zirconia beads were more efficient than larger diameter beads on lysing Gram-positive bacteria or even bacteria spores (Cohn et al., 1957; Käser, Ruf, Hauser, Marsollier, & Pluschke, 2009). In this perspective, downgrading the diameter and use a different material of beads could be a good solution. A good idea might be reutilizing the collected evaporate solvent, and use them again to perform a new extraction, permitting to save the use of new solvent mixture.

A detailed observation on the data concerning the VLC demonstrate that most of the recovered masses were in the last fractions, that is, the most polar ones. This leads to conclude most of the compounds that were extracted from the spores have a more affinity to polar solvents. Besides, since the last mobile phase was constituted by 100% MeOH, some silica from the stationary phase could be dissolved in the solvent, turning to have more mass than expected. (Edwards et al., 2004) Following the results of the gravitational chromatography performed on PTAFCES, it is possible to observe a losing of 28.05mg of this original mass, obtaining in the final of the process 105.35mg. Due to the interaction of the compounds with silica combined with a low velocity of the chromatography, some of the substance could be immobilized by the mobile phase (Swadesh, 2001; Wu, 2004). As similar to the others chromatography's, comparing the cytotoxicity assays from these fractions, it was verified that the active fractions possess very low masses. Given this, performing a purification of each fraction with such low masses, is challenging, risking some compounds to be retained in the stationary phase of the HPLC column and losing mass. As was describe before, the active fractions were collected into a single pool, hoping with this to obtain sufficient mass of the active compounds to perform its isolation. The chromatograph profile revealed this mixture to be too much complex to perform an efficient fractionation through HPLC technique. Increasing the VLC and HPLC resolution could be achieved by changing the polarity gradient more slowly, more specifically in the polarity region where are located the active compounds.

The NMR profile of PTACES showed that this fraction was too complex to verify the type of compounds present in the mixture. However, the presence of doublets at 7.90 δ , 7.59 δ and quartet 7.10 δ , demonstrated the possibility of similar compounds being present in the same fractions. In fact, these peaks were crucial along with the cytotoxic assays to decide

to collect the fraction between 12-19 in a single mixture to have sufficient mass, and so to perform a chromatography to isolate the present active compounds. Related with NMR analyses, a research on the literature revealed that the presence of the singlets at 7.36 δ and 3.49 δ are coincident with existence of chloroform and methanol in the fractions, respectively.(Fulmer et al., 2010). The presence of chloroform can be justified since the fractions were dissolved in this solvent to be subjected to an NMR analyses. Although, the presence of methanol was considered a contaminant, with a possible origin on deficient wash of the NMR tubes or even inadequate evaporation, resulting in traces of this solvent in the extracts.

The chromatograms confirm the results from NMR analysis. The collection of the fractions between 12-19 was a mixture of diverse compounds, revealing to be very challenging to perform isolation and identification with such low masses. The chromatogram A (Figure 16) with an isocratic mixture of 100% revealed that most of the compounds passed in the HPLC detector between 6:00 min and 21:00 min, in such way to be ineffective to perform the isolation of each substance. Yet, the NMR analysis suggested that some compounds share the same chemical template, which has led to group fractions that potentially contain different components. The complexity of this mixture of substances posed a challenge and questions if this procedure was the ideal. To minimize reaching to this dead end, it is imperative to increase the extraction efficiency, to recover more mass (more precisely in the active fractions), diminishing the possibility of losing mass and finally elaborate an appropriate method of fractioning, with a special emphasis on the polarity gradient corresponding the most active fractions.

The aim of performing a cell viability assay was with purpose of guide the fractionation process. In a general view, all the extractions possess compounds that can reduce the cellular viability. Firstly, it is important to assert that there are some variables that can influence the resolution of the chromatography and the time of retention in the stationary phase (ex. impurities, temperature, the operator skill etc.), proving the reproductive of this process being very difficult. In other words, the active compound can be present in a varied concentration in the different fractions according with the extract. These phenomena can be observed by the fact of the same fractions of E15094, PT01 and E16152, show different cellular viability, when even exposed to same cell lines and concentration. Besides this, a pattern is found when looking to the more bioactive fractions. The cell lines were more sensitive with the fractions between D and G, leading to conclude the presence of compounds with high ability to reduce the cellular viability. Comparing the impact of the cell viability in the cancer and normal cells, it is verified that they are both affected by the active compounds. Although, some fraction showed to be

more toxic to the cancer cells than the normal ones. Observing the cell viability data obtained in the fraction E from E16152, the HEPG2 cells are clearly more affected than the HCMEC/D3 after a treatment of 1 µg /ml with 72 h of exposure. The same effects are evident, by spotting the toxicity induced by the fraction 8 in the HCMEC/D3 and HEPG2 cell lines. The absence of toxic impact in the normal cell, contrasting with the low cellular viability in cancer lines, proposes the possibility of the presence of active compounds with selectivity toward HEPG2. The results corroborate the data presented by (Alves et al., 2015), when the author suggest that the active compounds could be more toxic to the cancer cells. The other explanation for this difference of cell viability between cancer and normal cells is the metabolic rate. The cancer cells are well known for having a higher metabolic rate, with a high glucose consumption to boost tumor proliferation, through a phenomenon designated Warburg effect. (Cairns, Harris, & Mak, 2011; DeBerardinis, 2008). Giving this, the cancer lines can absorb more nutrients (including the bioactive compounds) and so, present the toxic effects early and more acutely. The percentage of viable cells was obtained through an MTT assay. Some author question if the results obtained through this colorimetric test could be considered reliable. The reduction of MTT into its formazan is dependable on cell metabolic rate. Consequently, cells that divide slowly will exhibit slow MTT reduction rates, when on other hand, cells with a high metabolism will reduce the MTT more faster. (van Tonder, Joubert, & Cromarty, 2015). Meanwhile, MTT test is a good assay for preliminary screening for bioactive compounds (as was design in this study) and to detect a direct impact in the cell metabolic activity. However, with the limitations of test, the toxicity of the bioactive compounds should be confirmed with other assay as lactate dehydrogenase (LDH) test, providing information about the integrity of the cellular membranes.

6. Conclusion

As referred, there has been a big demand for the search of new compounds, that can be key to fight illness, such as cancer or obesity or problematic issues as the microbial resistance to the antibiotics. With this, fungi emerge as great sources of bioactive compounds for giving response to these challenging issues, as solution to other biomedical topics. Giving the lack of data about the bioactive potential of spores of *P. tinctorius*, Alves et al. (2015) opened a new chapter by confirming the existence of compounds in this structures able to affect the cell viability, and with a remarkable cytotoxicity in the cancer cell lines. However, giving the natures of the spores as resistance structures, it was difficult to perform an extraction with a substantial recovery of mass. The study conducted on this thesis tried to give an answer to the low efficiency extraction problem, and at the same time attempt to obtain more data about the bioactive compounds present in the spores. The results obtained confirm that the spores of *P. tinctorius* can be a useful source of bioactive compounds, more specifically, with anticancer potential. Although it was not possible to identify the compound(s) responsible for this cytotoxicity, the extraction method was improved, confirming that the glass beads are a useful tool to force the lysis of strong structures, opening a door for upgrades in this technique, since it is imperative to improve the extraction yield. However, further research efforts must be conducted to identify and isolate the compounds responsible for cytotoxicity effect and respective mechanisms of action. Giving this anticancer effect, it would be interesting to study the spores as a potential source for new compounds with bioactive properties in different fields (ex. anti-inflammatory, anti-microbial, etc.).

7. Appendixes

7.1 Appendix A- ^1H NMR data for sub-fractions resulting from PTAFCES.

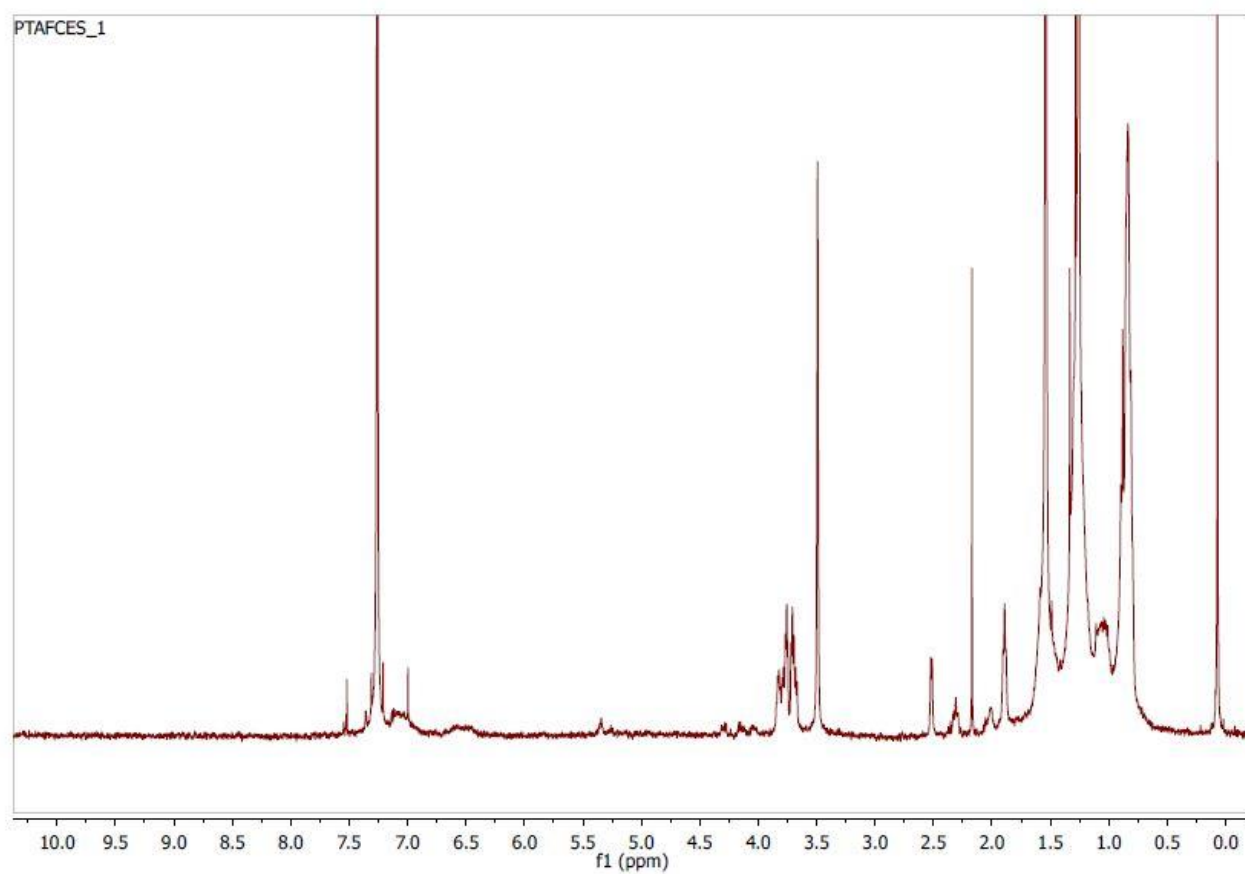


Figure 1. NMR spectrum from fraction 1 of PTAFCES in CDCl₃ (recorded at 600 MHz)

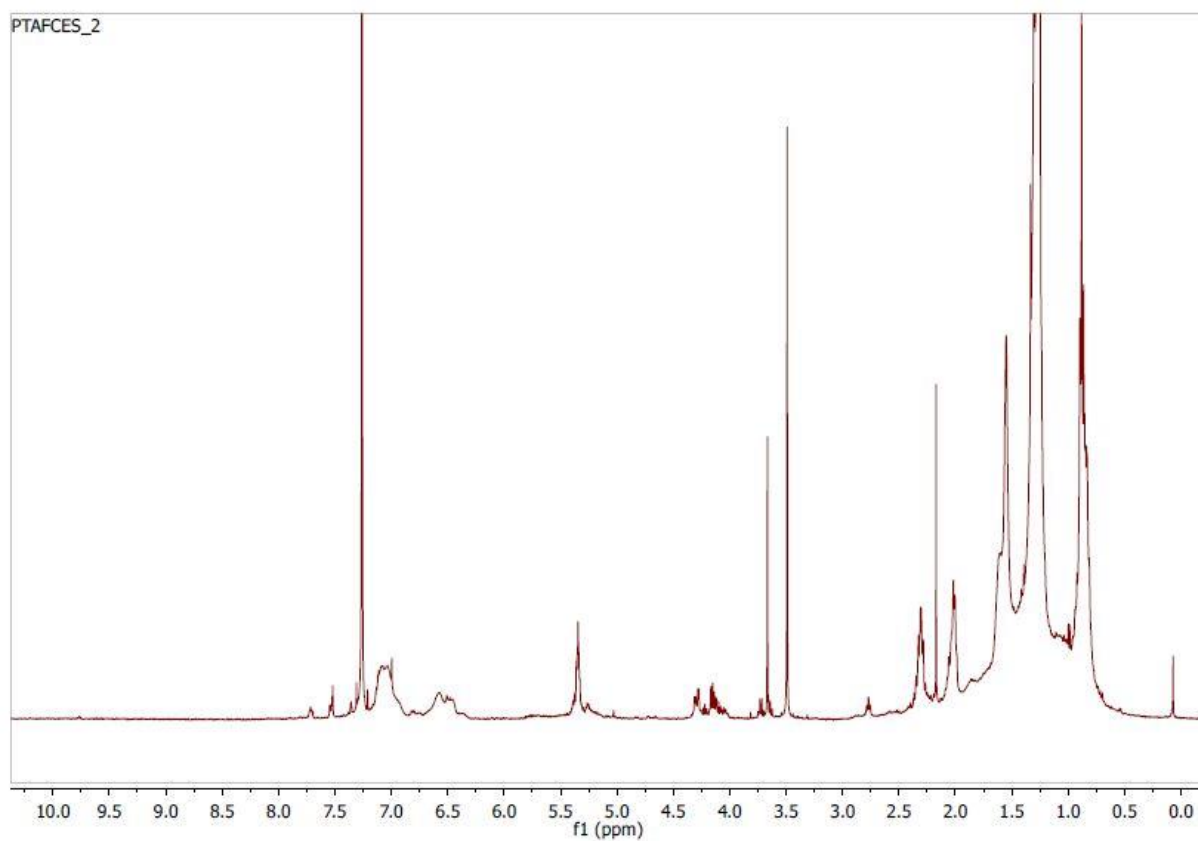


Figure 2. NMR spectrum from fraction 2 of PTAFCES in CDCl_3 (recorded at 600 MHz)

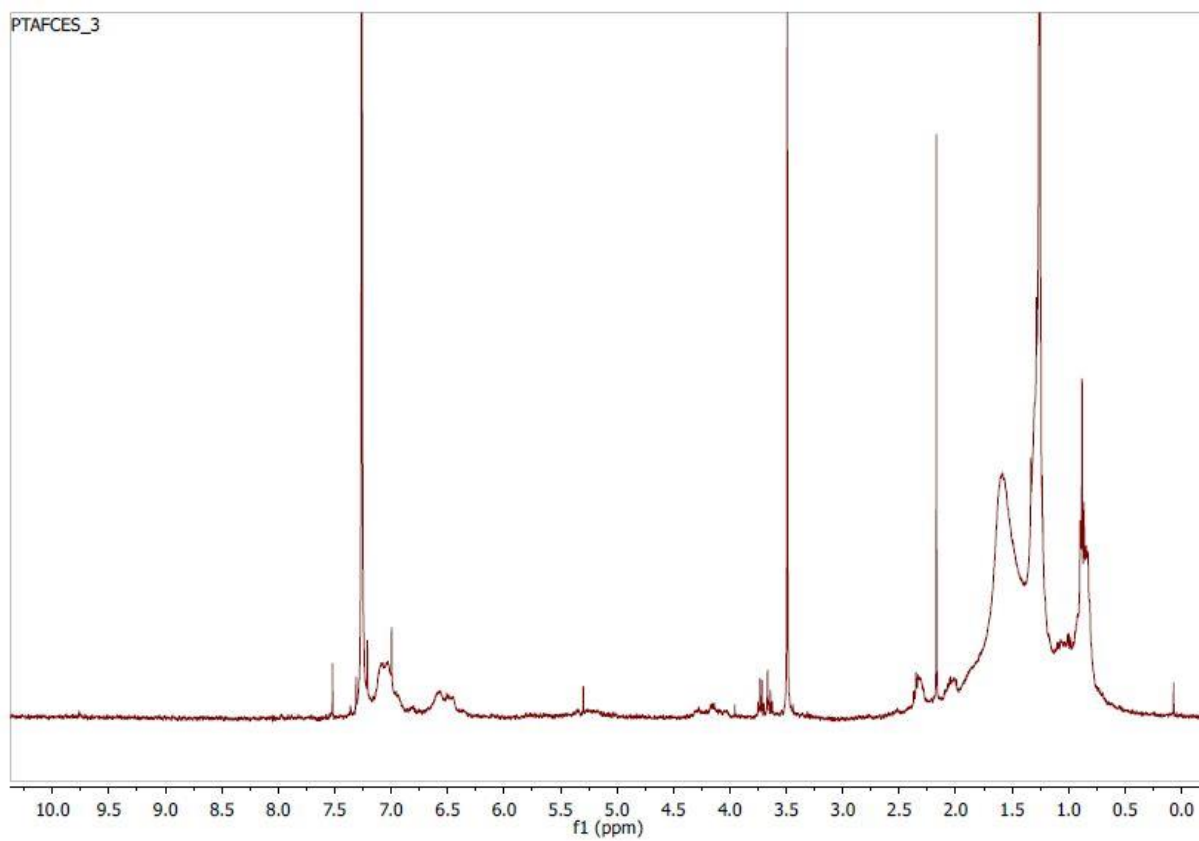


Figure 3. NMR spectrum from fraction 3 of PTAFCES in CDCl_3 (recorded at 600 MHz)

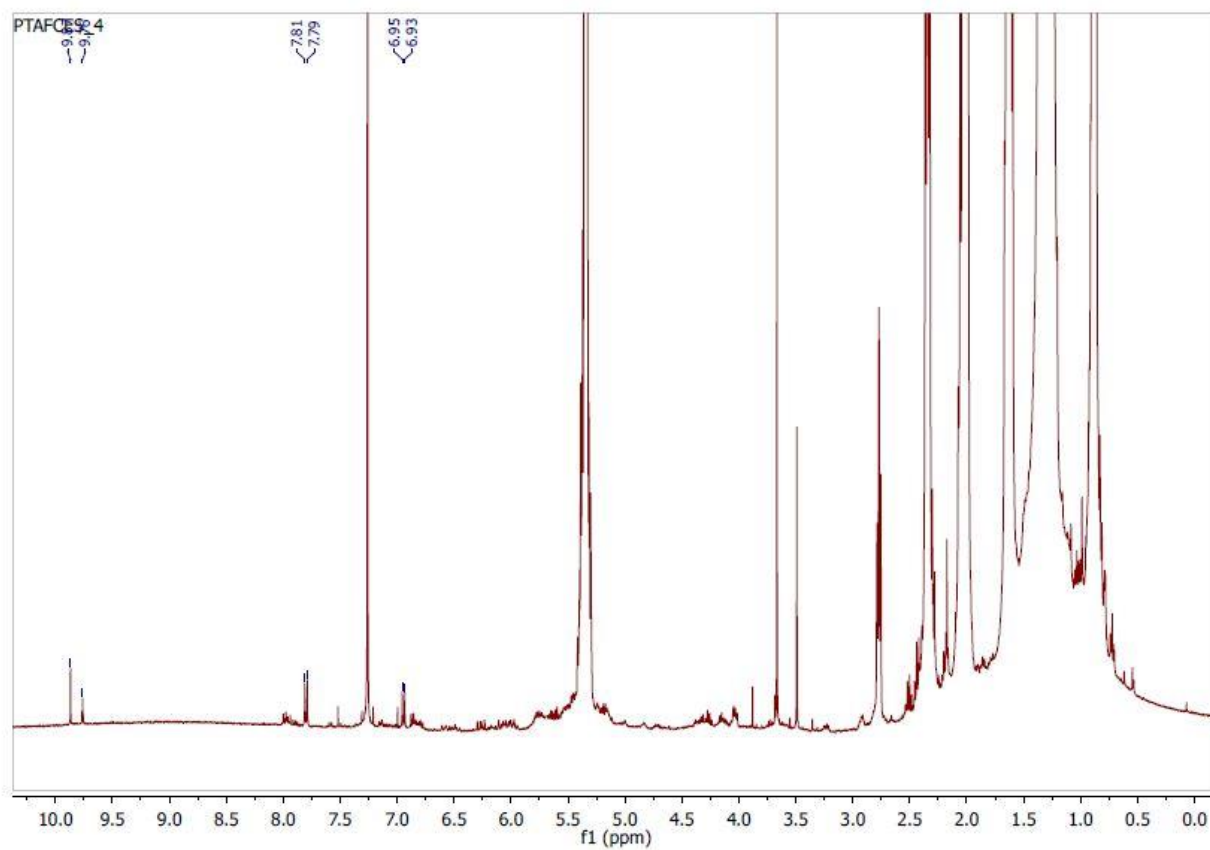


Figure 4. NMR spectrum from fraction 4 of PTAFCES in CDCl_3 (recorded at 600 MHz)

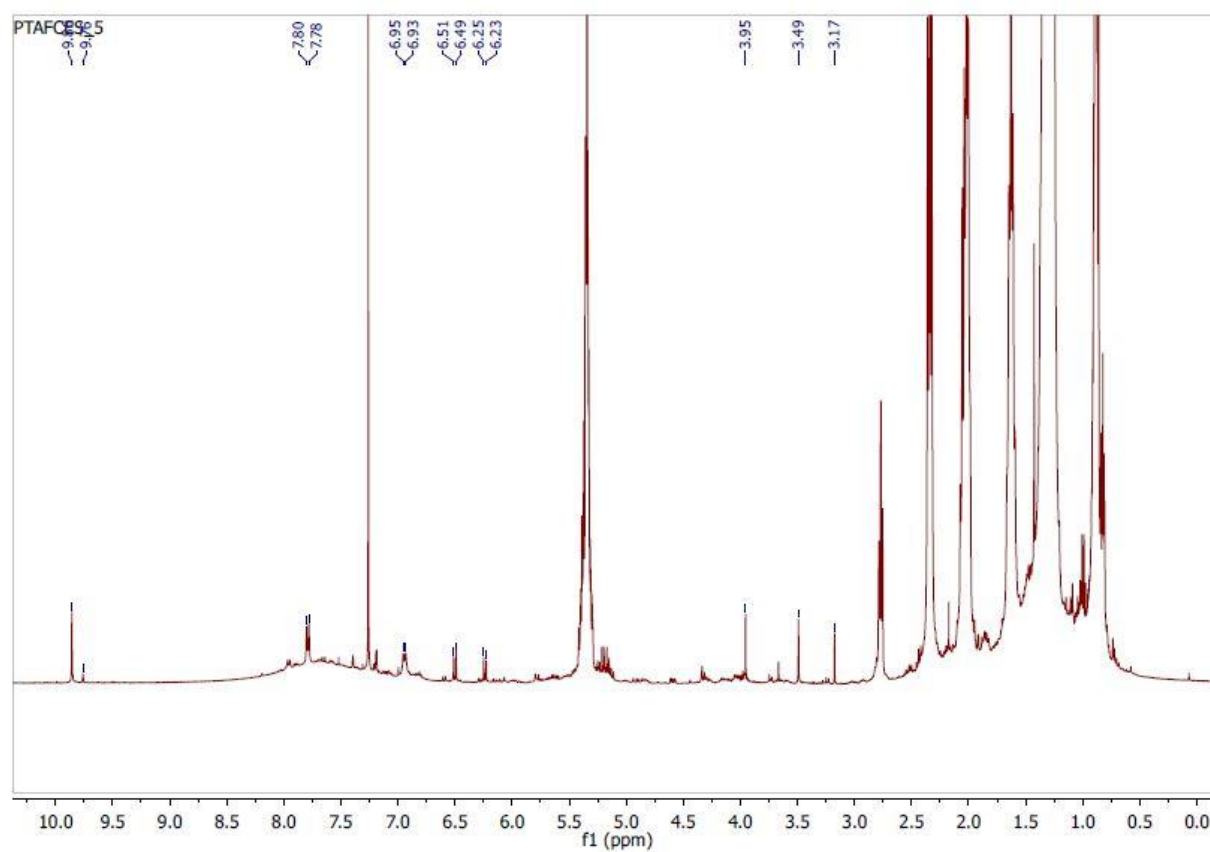


Figure 5. NMR spectrum from fraction 5 of PTAFCES in CDCl_3 (recorded at 600 MHz)

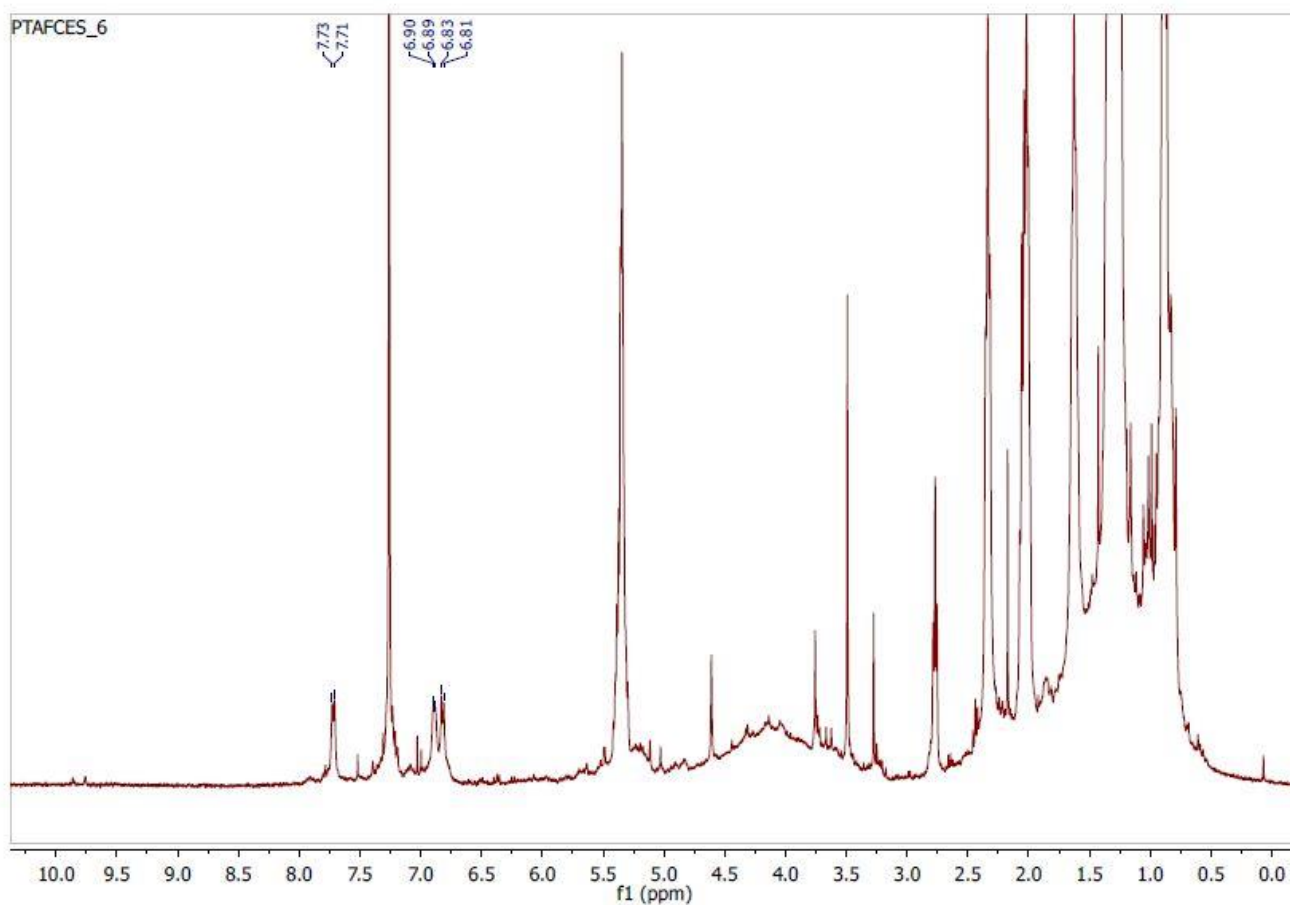


Figure 6. NMR spectrum from fraction 6 of PTAFCES in CDCl_3 (recorded at 600 MHz)

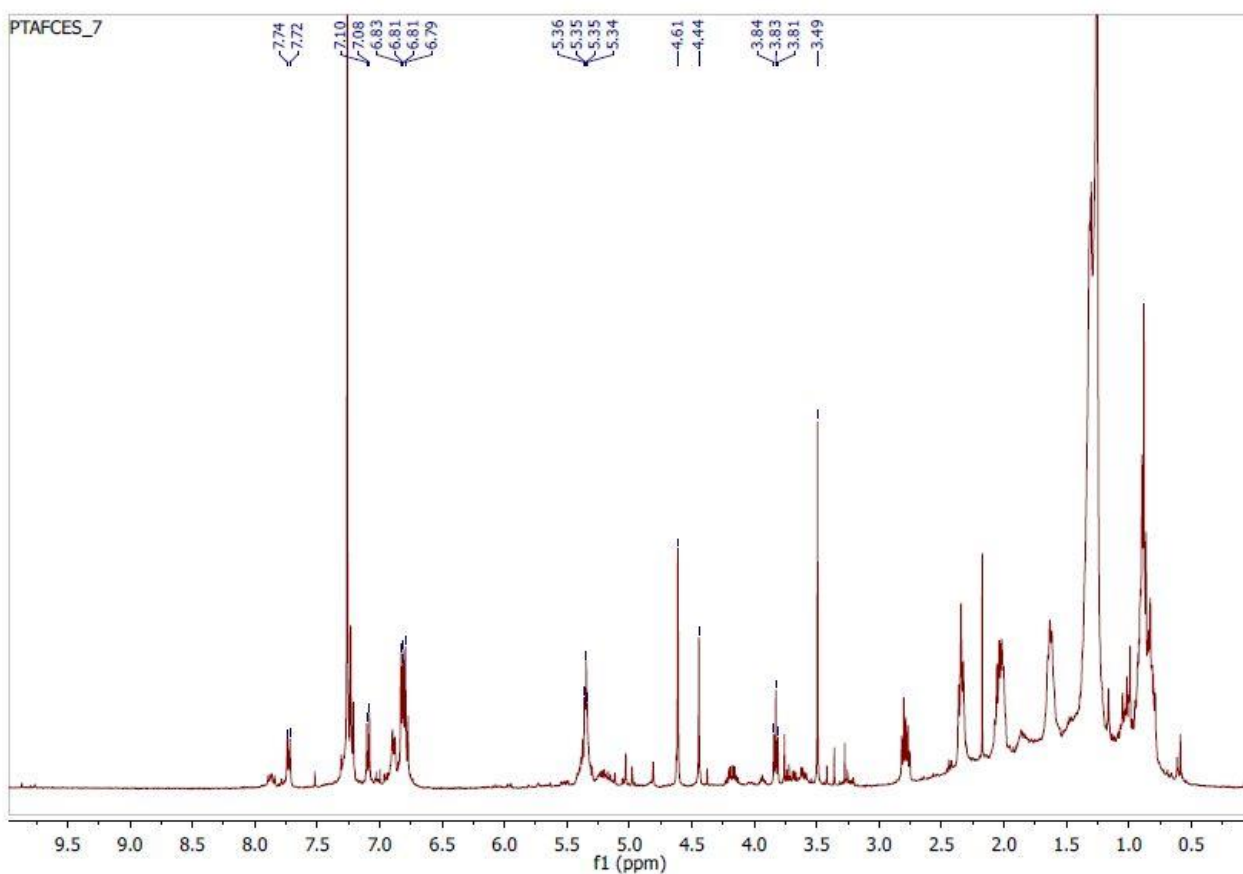


Figure 7. NMR spectrum from fraction 7 of PTAFCES in CDCl_3 (recorded at 600 MHz)

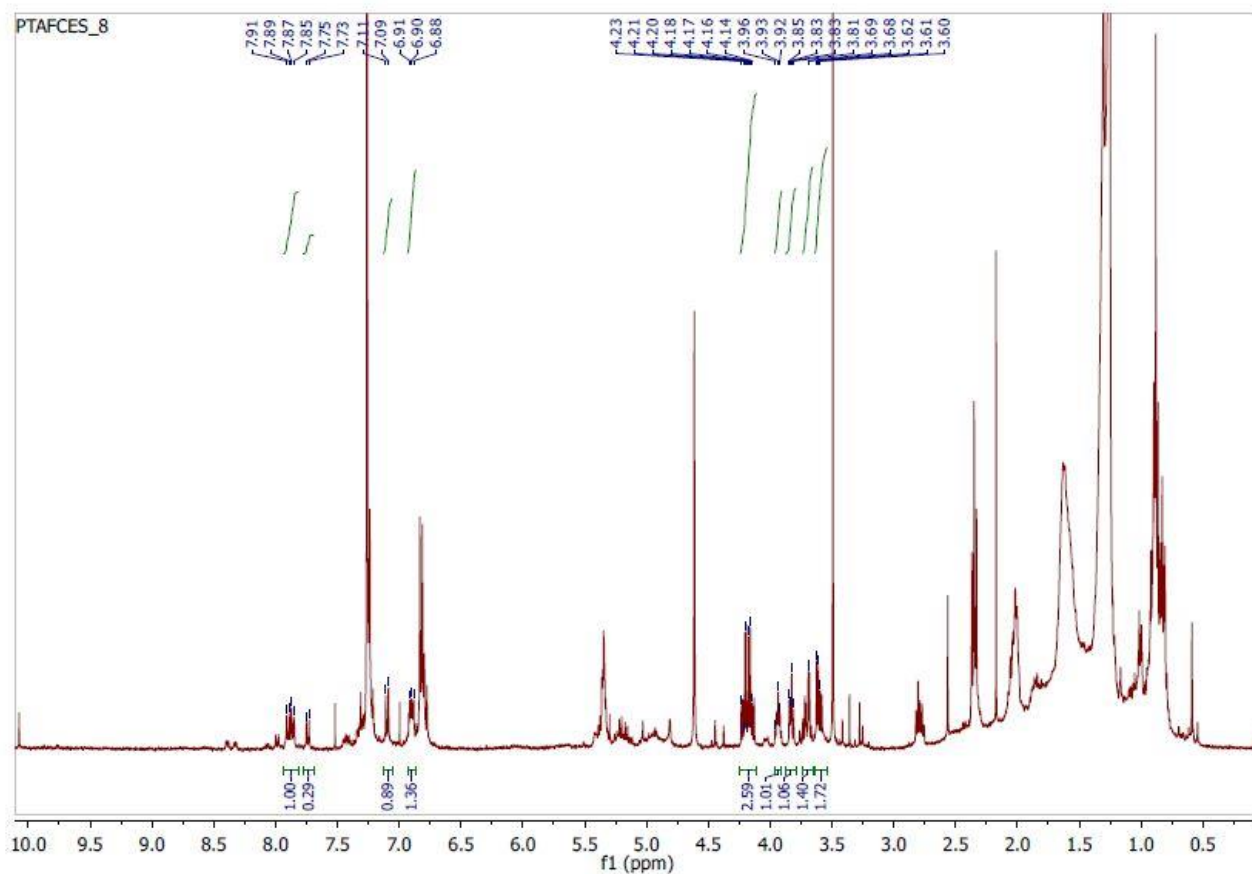


Figure 8. NMR spectrum from fraction 8 of PTAFCES in CDCl_3 (recorded at 600 MHz)

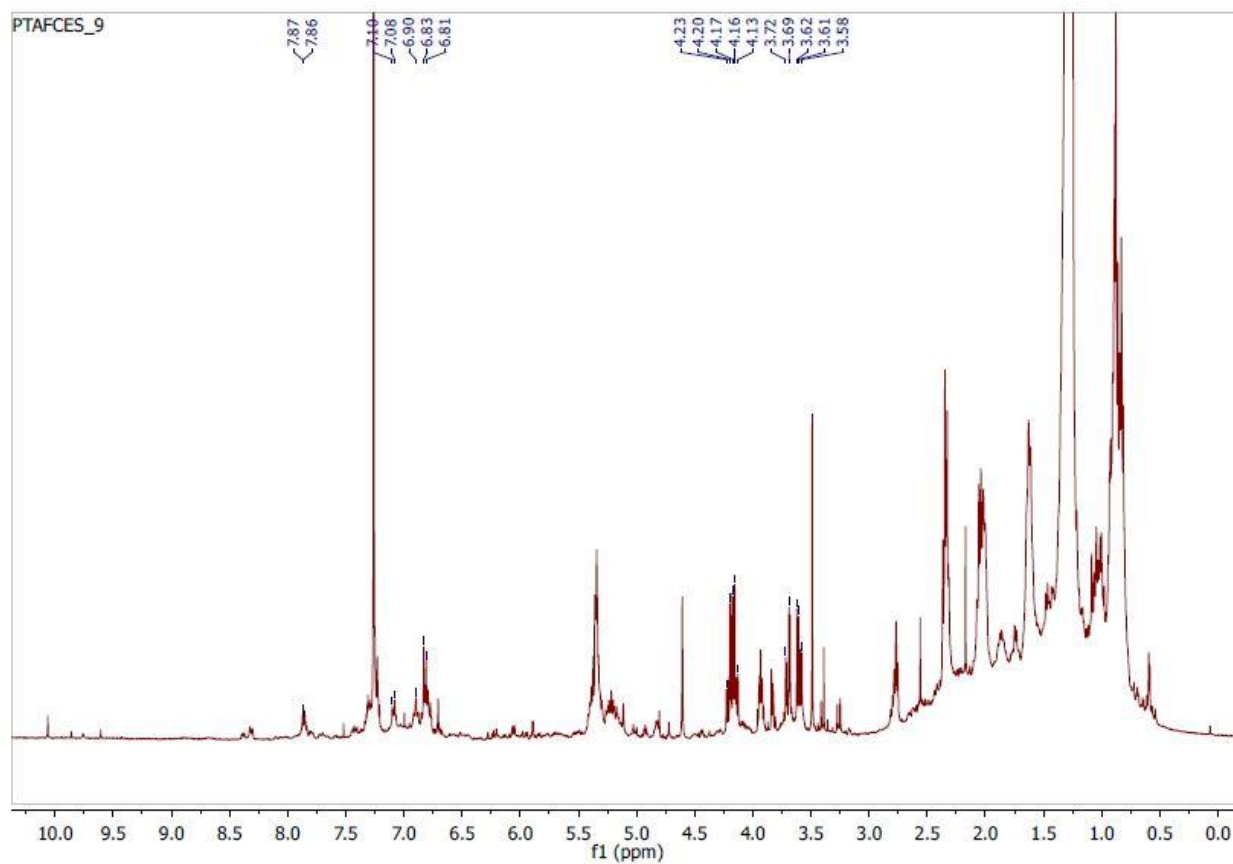


Figure 9. NMR spectrum from fraction 9 of PTAFCES in CDCl_3 (recorded at 600 MHz)

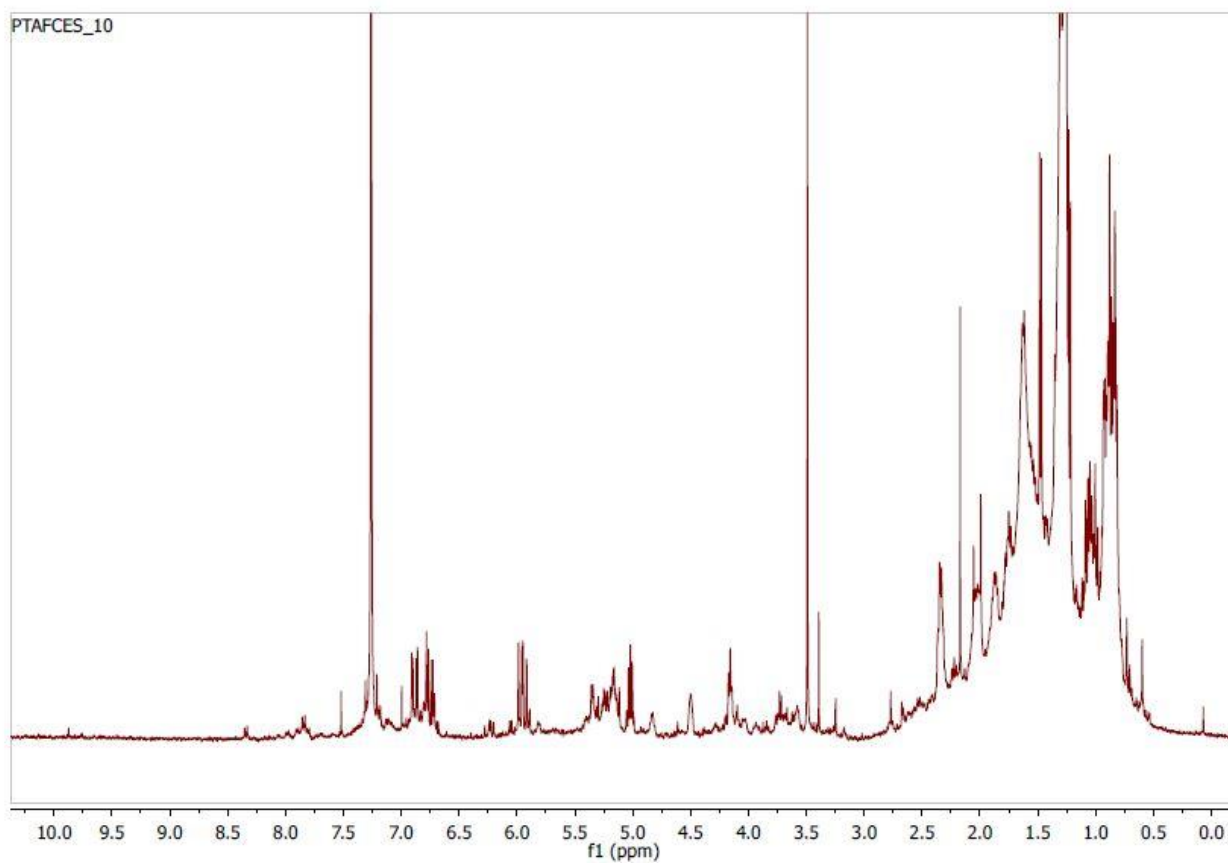


Figure 10. NMR spectrum from fraction 10 of PTAFCES in CDCl_3 (recorded at 600 MHz)

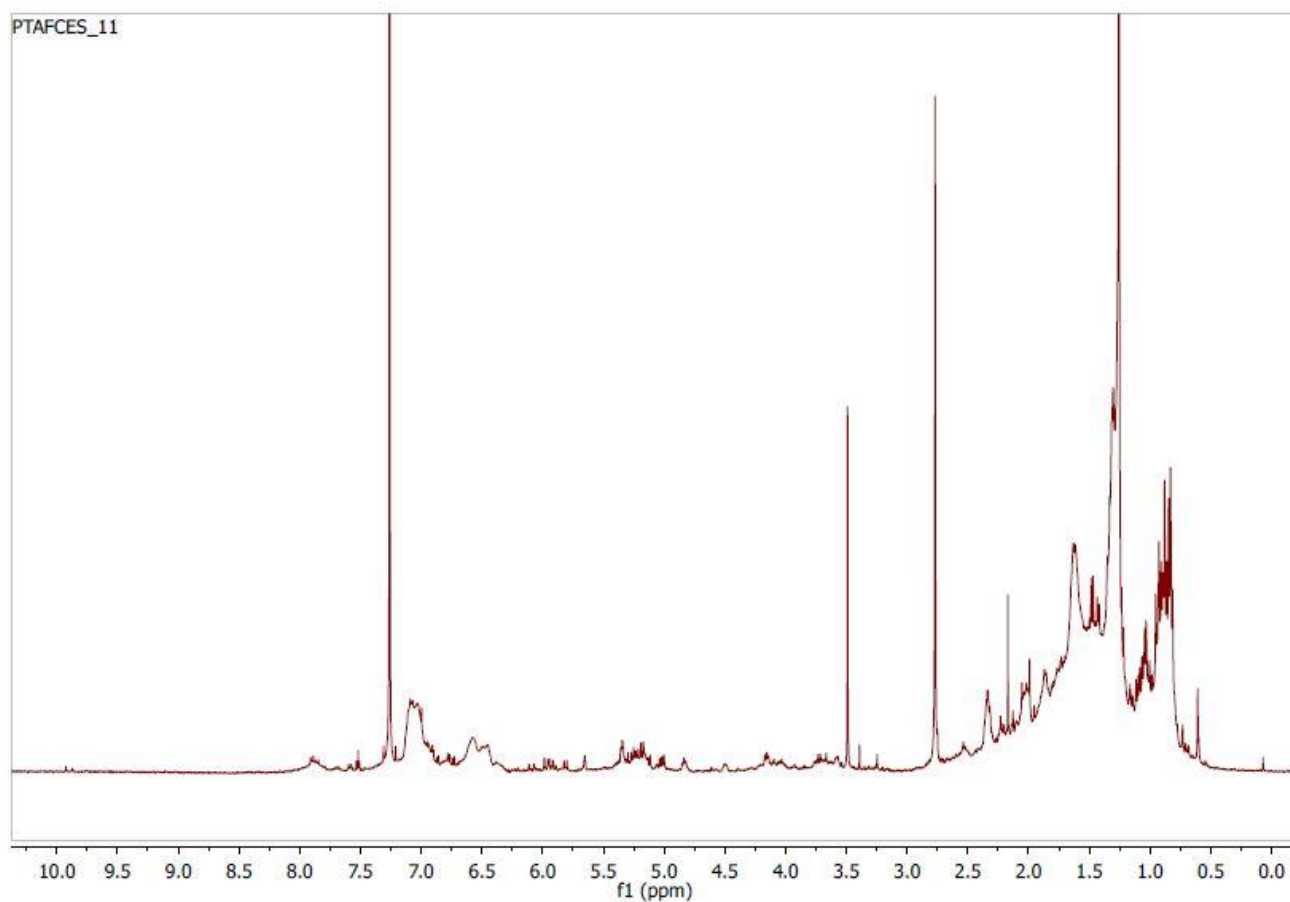


Figure 11. NMR spectrum from fraction 11 of PTAFCES in CDCl_3 (recorded at 600 MHz)

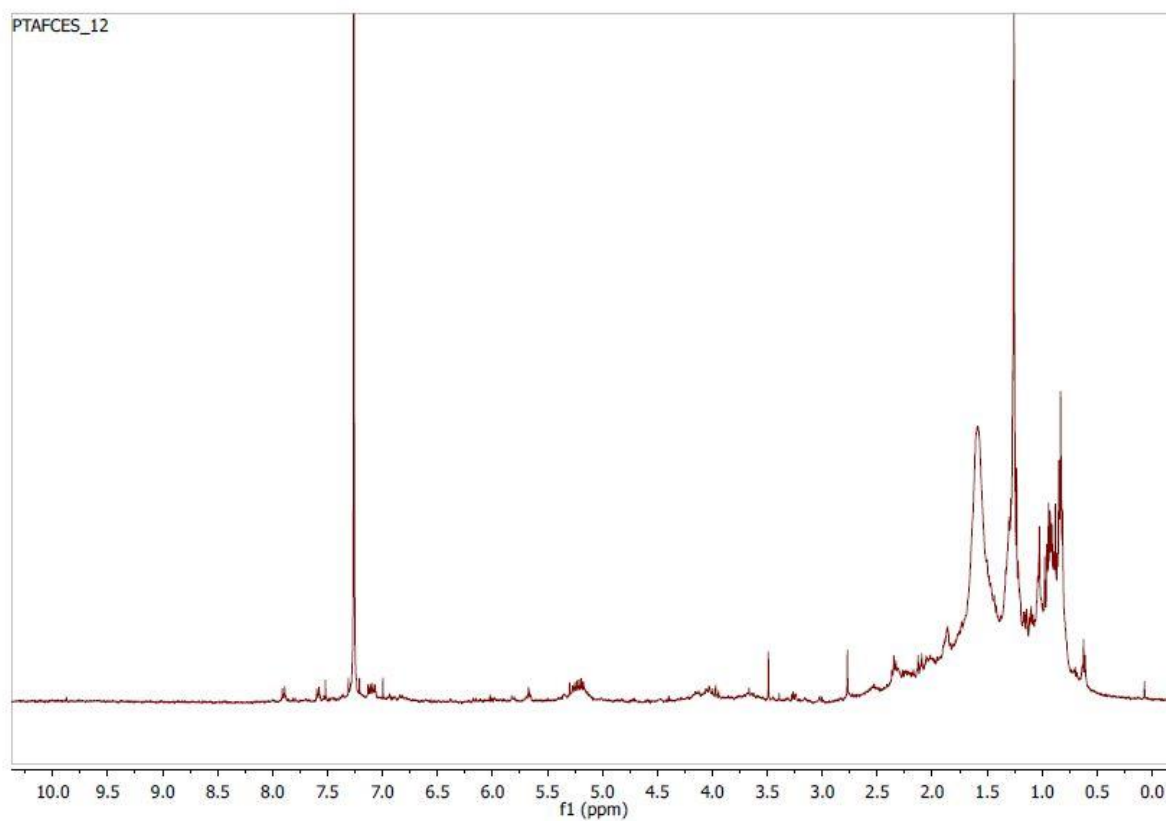


Figure 12. NMR spectrum from fraction 12 of PTAFCES in CDCl_3 (recorded at 600 MHz)

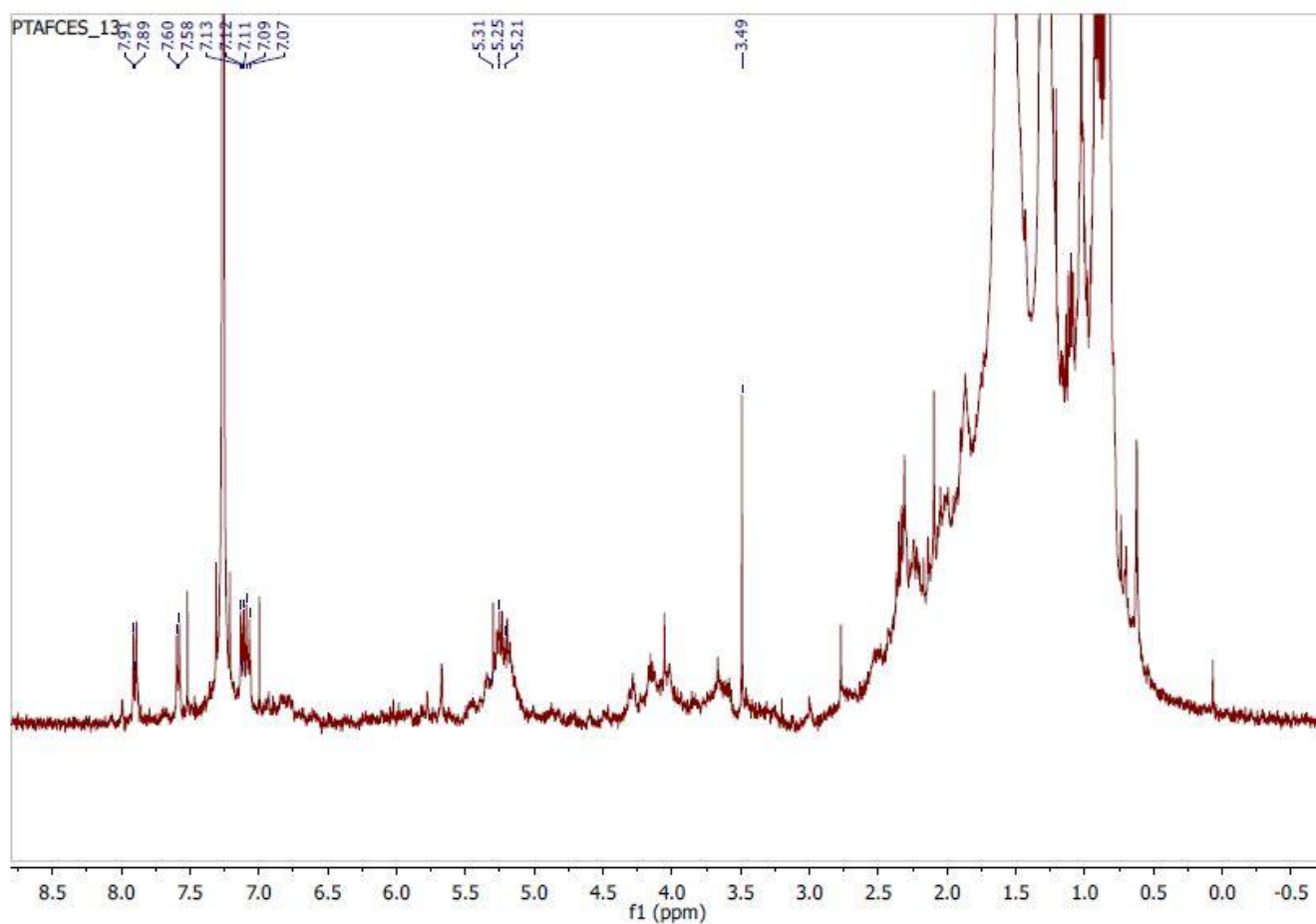


Figure 13. NMR spectrum from fraction 13 of PTAFCES in CDCl_3 (recorded at 600 MHz)

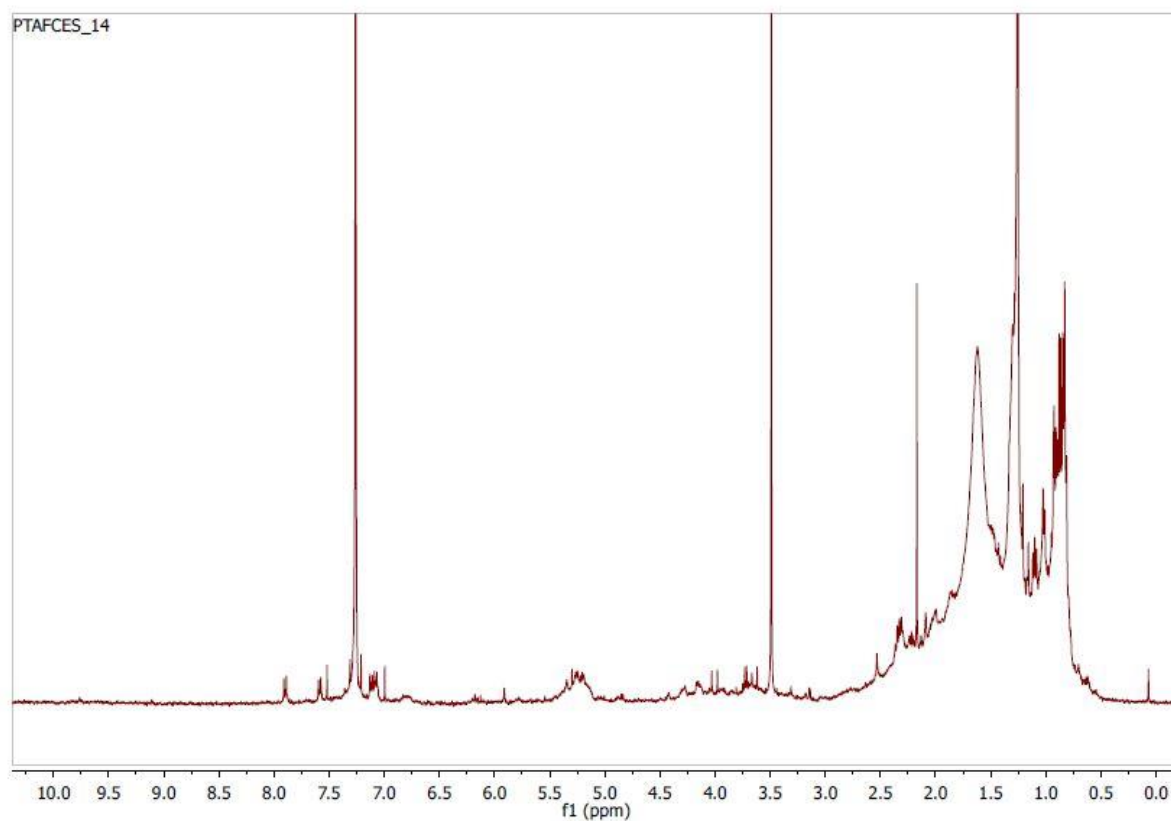


Figure 14. NMR spectrum from fraction 14 of PTAFCES in CDCl_3 (recorded at 600 MHz)

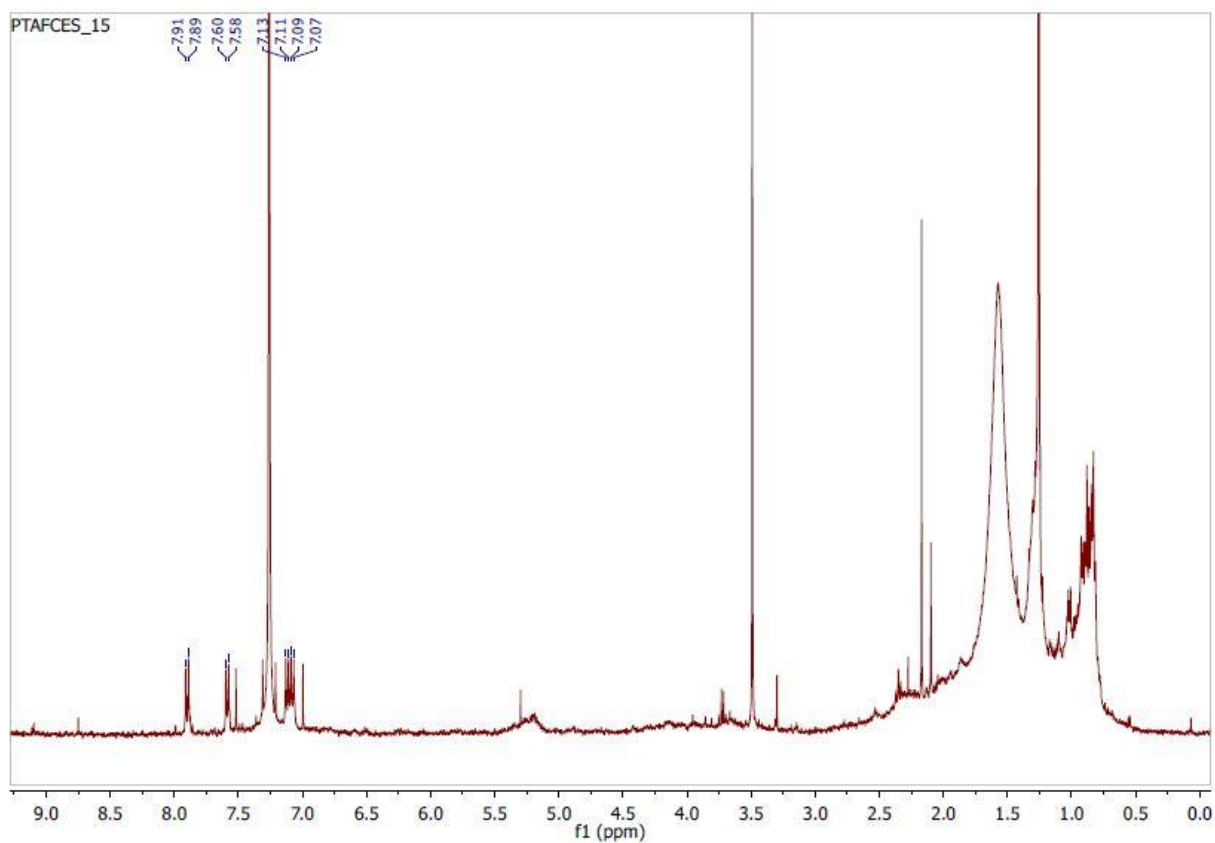


Figure 15. NMR spectrum from fraction 15 of PTAFCES in CDCl_3 (recorded at 600 MHz)

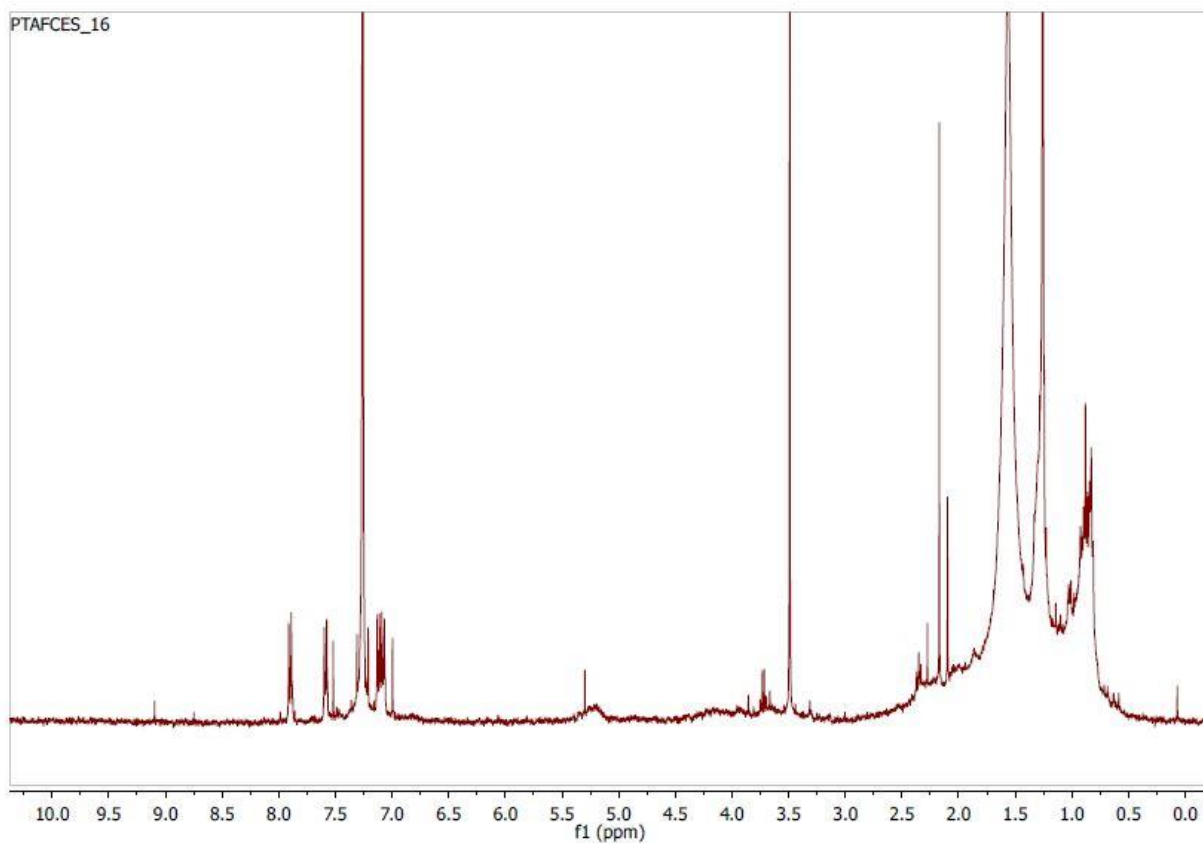


Figure 16 NMR spectrum from fraction 16 of PTAFCES in CDCl_3 (recorded at 600 MHz)

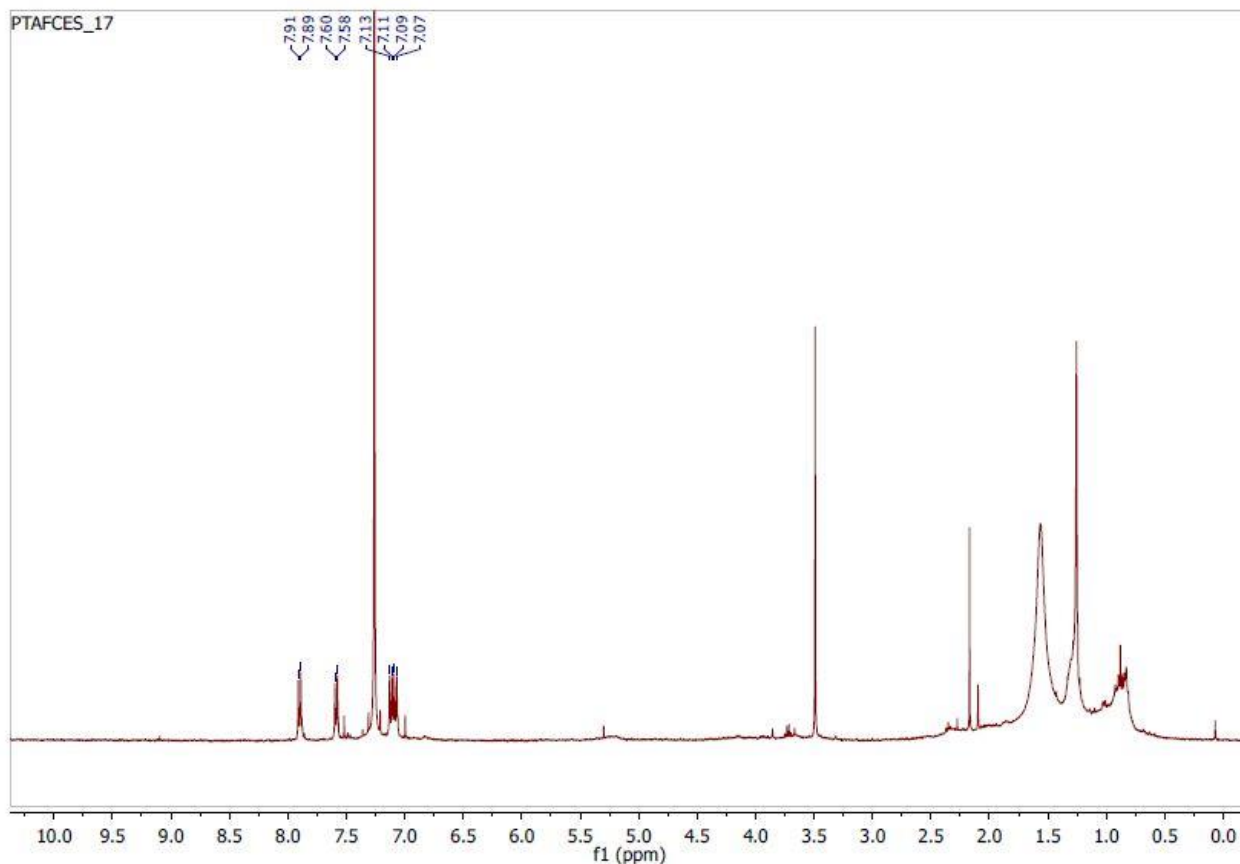


Figure 17 NMR spectrum from fraction 17 of PTAFCES in CDCl_3 (recorded at 600 MHz)

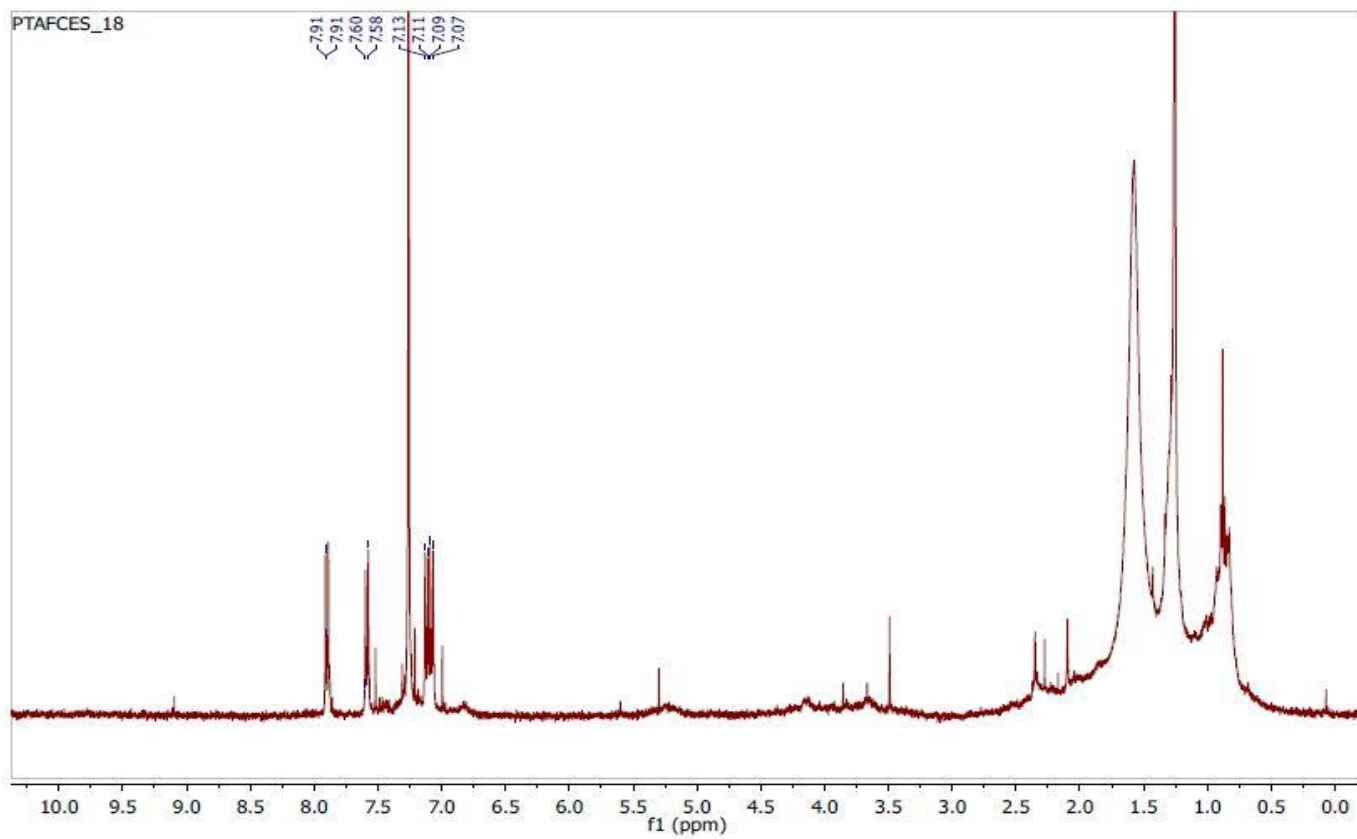


Figure 18 NMR spectrum from fraction 18 of PTAFCES in CDCl_3 (recorded at 600 MHz)

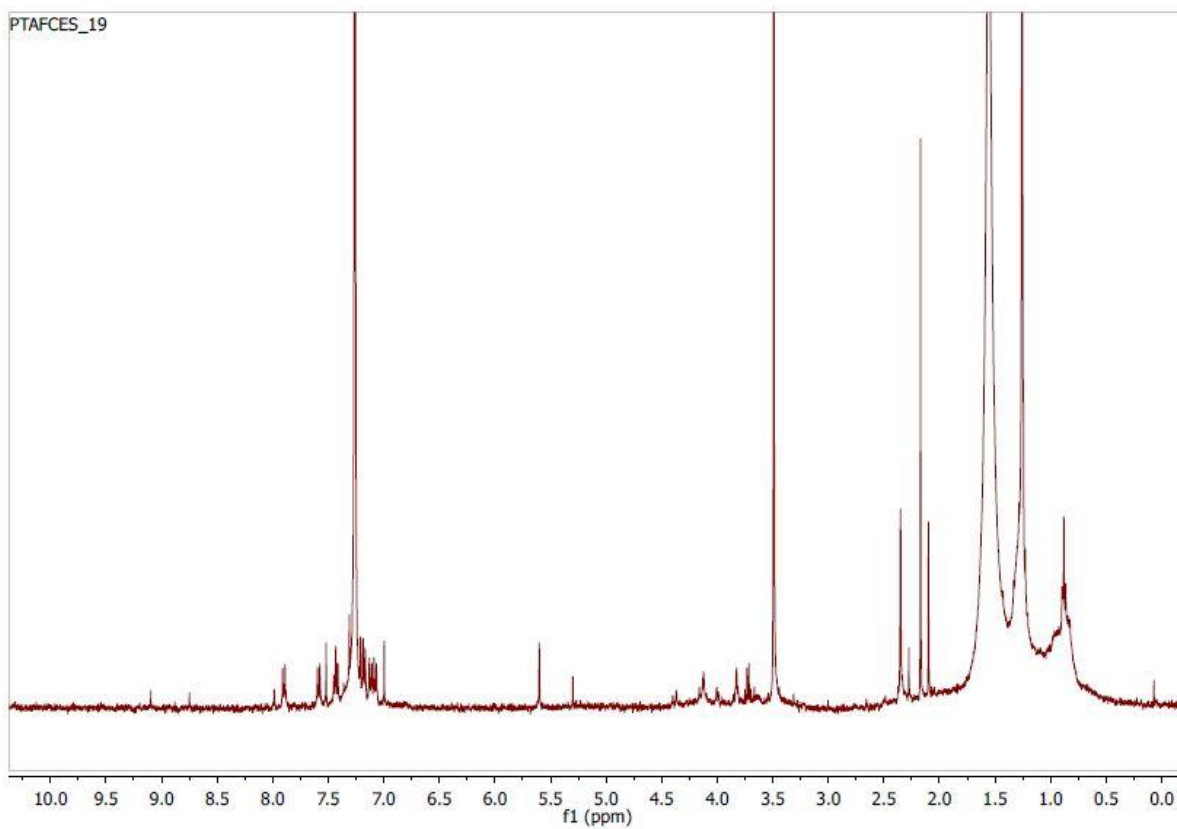


Figure 19 NMR spectrum from fraction 19 of PTAFCES in CDCl_3 (recorded at 600 MHz)

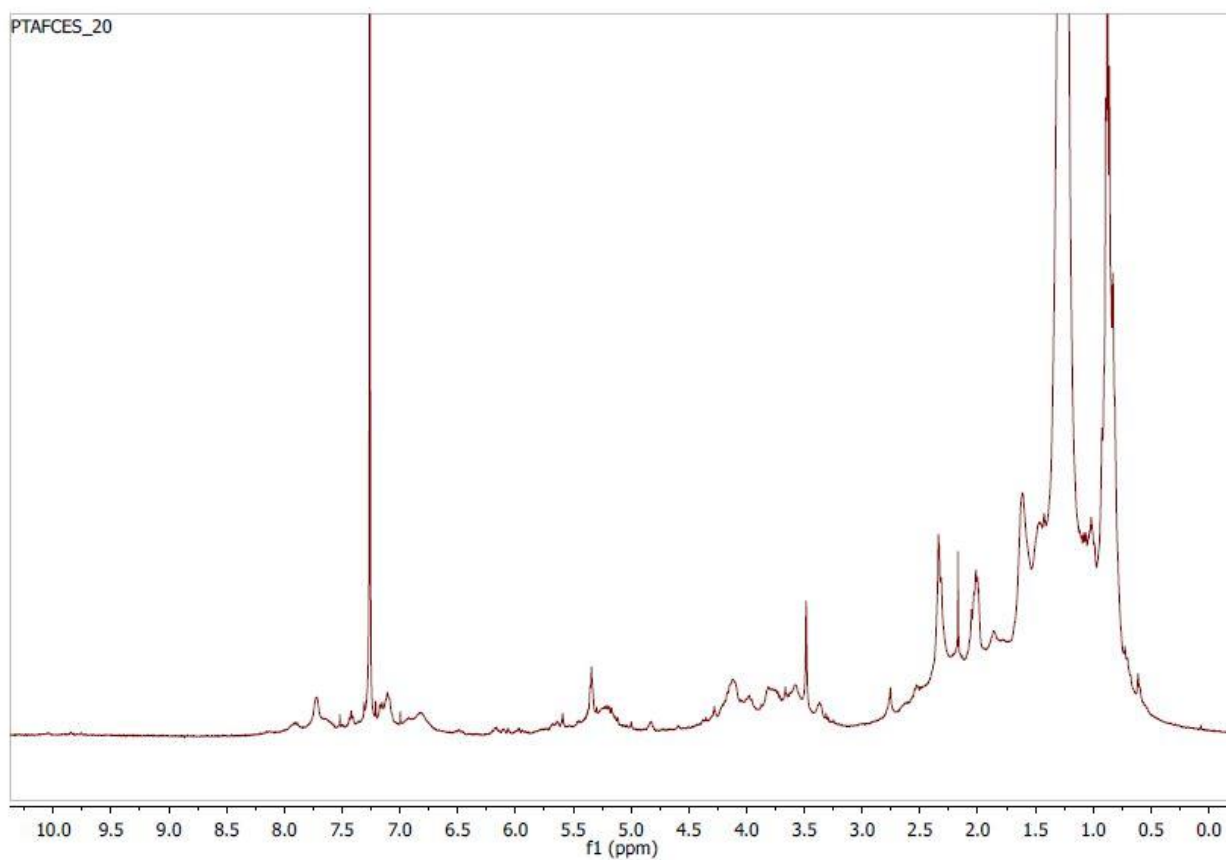


Figure 20 NMR spectrum from fraction 20 of PTAFCES in CDCl_3 (recorded at 600 MHz)

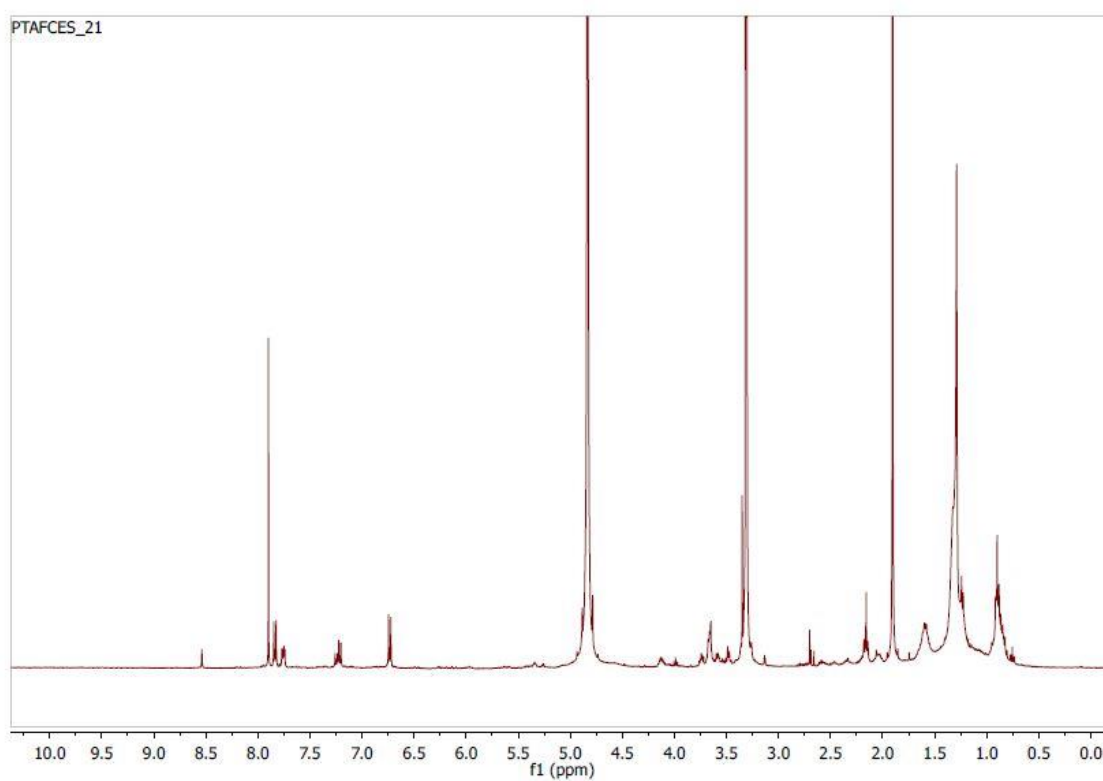


Figure 21 NMR spectrum from fraction 21 of PTAFCES in CDCl_3 (recorded at 600 MHz)

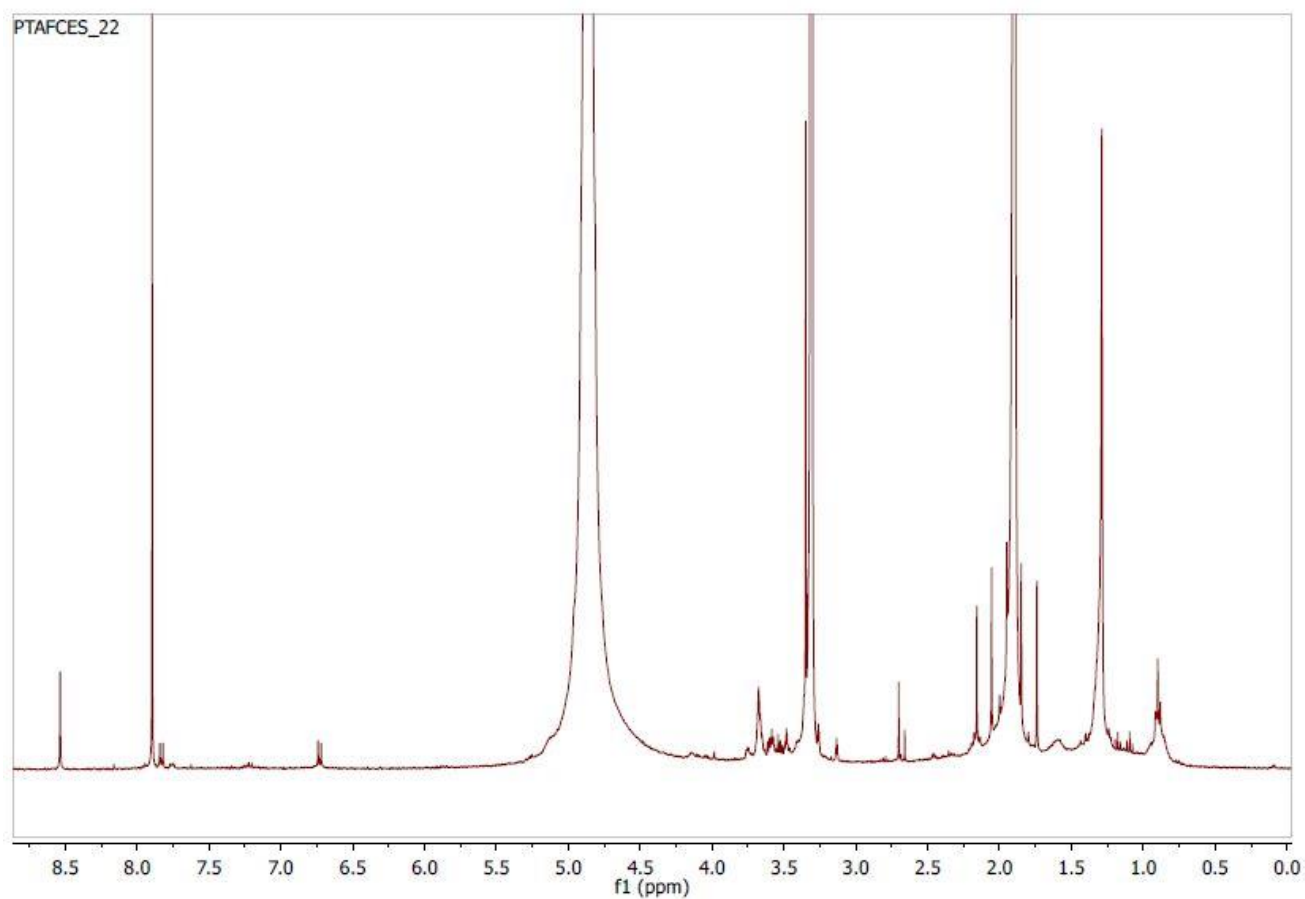


Figure 22 NMR spectrum from fraction 22 of PTAFCES in CDCl_3 (recorded at 600 MHz)

7.2 Appendix B - Effects of fractions from E16151 at 20µg/ml in the viability of HEPG2 (A) and HDMC (B)

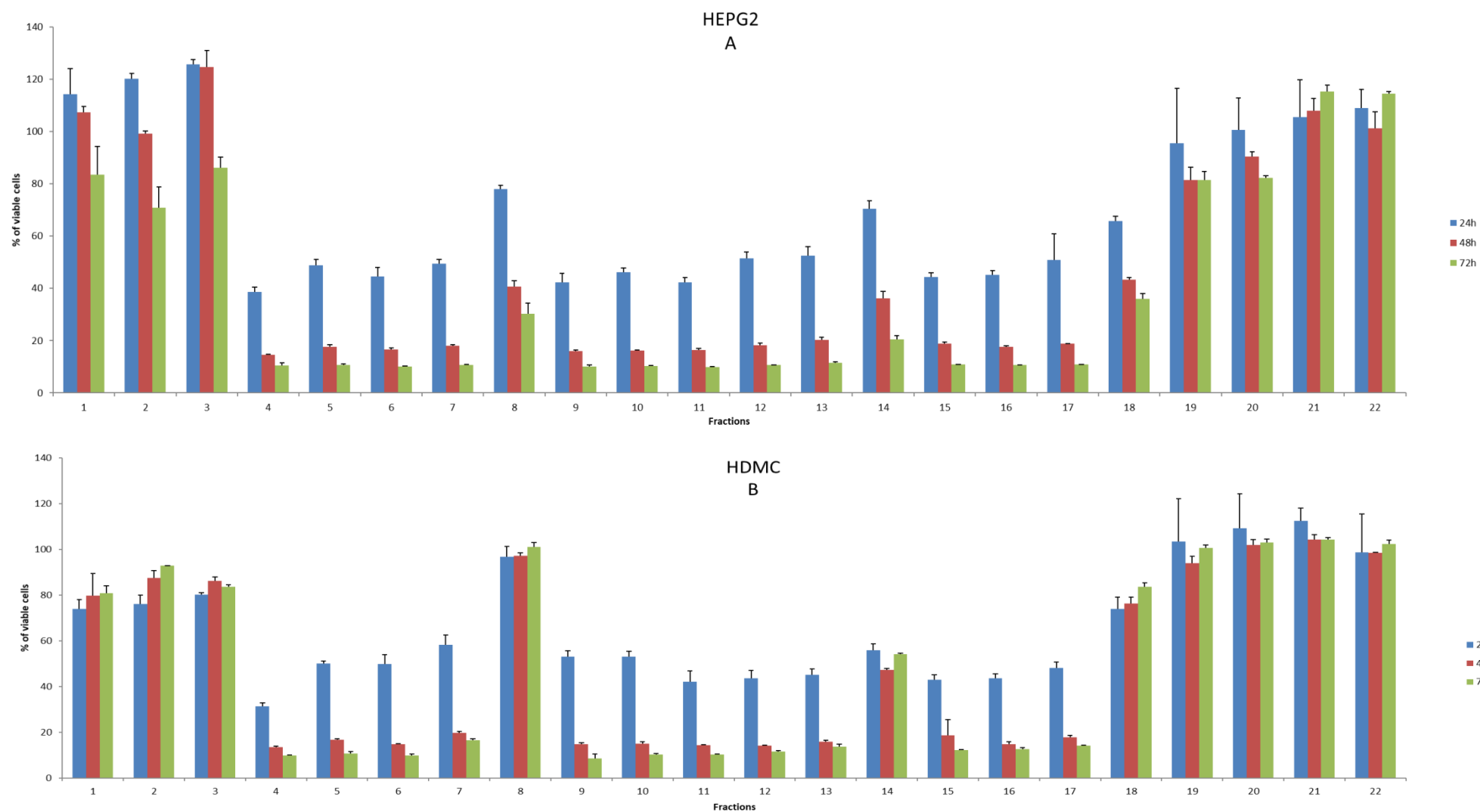


Figure 15. Effects of fractions from PTAFCES at 20µg/ml in the viability of HEPG2 (A) and HDMC (B). Values are present as means \pm SD of triplicate analyses when compared with control group (n = 3).

8. References

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